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ROLE OF MITOCHONDRIAL RIBOSOMAL PROTEIN S18-2 IN CANCEROGENESIS AND IN REGULATION OF STEMNESS AND DIFFERENTIATION

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Role of Mitochondrial Ribosomal Protein S18-2 in Cancerogenesis and in Regulation of Stemness and Differentiation
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to my parents

ABSTRACT

Mitochondria carry their own ribosomes (mitoribosomes) for the translation of mRNA encoded by mitochondrial DNA. The architecture of mitoribosomes is mainly composed of mitochondrial ribosomal proteins (MRPs), which are encoded by nuclear genomic DNA. Emerging experimental evidences reveal that several MRPs are multifunctional and they exhibit important extra-mitochondrial functions, such as involvement in apoptosis, protein biosynthesis and signal transduction. Dysregulations of the MRPs are associated with severe pathological conditions, including cancer. Cancer cells are immortal, i.e. they proliferate in limitless mode, avoiding cell cycle control which is a characteristic of normal cells. Retinoblastoma susceptibility (RB) protein is the major regulator of cell cycle and it operates by inhibiting the activity of E2F1 transcription factor through direct binding.

This doctoral thesis is devoted to the extra-mitochondrial role of a RB interacting MRP, the MRP-S18-2 (or S18-2), in different aspects of cancer. S18-2 is localized to the small subunit of mitoribosome and it belongs to S18 family of MRPs, a family consisting of three proteins. Previously, it was shown that S18-2 binds to RB, inhibiting the association of the latter with a transcription factor E2F1 that regulates G_1/S transition of cell cycle. Overexpression of S18-2 in rat embryonic fibroblasts led to their immortalization with induction of stem cells markers.

In this thesis, I describe that overexpression of S18-2 can also immortalize terminally differentiated rat skin fibroblasts. The immortalized cells displayed characteristics of transformed cells with severe chromosomal instability, disrupted cell cycle, enhanced telomerase activity and the ability to produce tumors in experimental animals. We also showed that rodent cells immortalized by S18-2 overexpression were targeted by NK cells mediated cytotoxicity.

Phylogenetic analysis of S18 proteins revealed specific gene duplication events that resulted in three S18 homologs in metazoan. In addition, a Gly132 polymorphism in S18-2 was observed in colon adenocarcinoma that was confirmed by PCR analysis and direct DNA sequencing.

The S18-2 protein level was increased, along with free E2F1, in endometrial cancer (EC). Moreover, high S18-2 levels may be associated with epithelial to mesenchymal transition in EC cells. Elevated levels of S18-2 were also found in prostate cancer (PCa). S18-2 could induce the CXCR4 mediated migration of PCa cells *in vitro* and in a zebrafish model. We also demonstrated new functional consequence of RB and S18-2 interaction in maintenance of a stem cell phenotype, using *RB1* knockout mouse embryonic fibroblasts. A cytoplasmic protein complex between S18-2, RB and the Ring finger protein 2 (RNF2) was detected. This enhanced the E3 ligase activity of RNF2, thus, maintaining cell stemness.

Our data supports and provide evidence to suggest that S18-2, a RB interacting protein, plays important roles in the development of cancer as a potent oncoprotein.

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I. Darekar SD, **Mushtaq M,** Gurrapu S, Kovalevska L, Drummond C, Petruchek M, Tirinato L, Di Fabrizio E, Carbone E, Kashuba E. 2015. Mitochondrial ribosomal protein S18-2 evokes chromosomal instability and transforms primary rat skin fibroblasts. Oncotarget 6(25):21016-21028.

II. **Mushtaq M**, Ali RH, Kashuba V, Klein G, Kashuba E. 2016. S18 family of mitochondrial ribosomal proteins: evolutionary history and Gly132 polymorphism in colon carcinoma. Oncotarget 7(34):55649-55662.

III. Mints M*, **Mushtaq M*,** Iurchenko N, Kovalevska L, Stip MC, Budnikova D, Andersson S, Polischuk L, Buchynska L, Kashuba E. 2016. Mitochondrial ribosomal protein S18-2 is highly expressed in endometrial cancers along with free E2F1. Oncotarget 7(16):22150-22158.

IV. **Mushtaq M#,** Lasse J, Davidsson S, Grygoruk A, Ove A, Kashuba V, Kashuba E#. The expression of MRPS18-2 increases with progression of prostate cancer inducing CXCR4 dependent cell migration. (Manuscript submitted to Scientific Reports).

V. **Mushtaq M,** Kovalevska L, Darekar SD, Abramsson A, Zetterberg H, Arsenian-Henriksson M, Kashuba V, Klein G, Kashuba E. Maintenance of the stem cell phenotype requires high expression levels of MRPS18-2 and presence of the RB protein. (Manuscript submitted to Plos Biology).

VI. **Mushtaq M**, Pangigadde PN, Darekar SD, Dissen E, Kashuba E. Rat embryonic fibroblasts immortalized by MRPS18-2 protein are targets for NK-cells. (Manuscript 2017).

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I. **Mushtaq M**, Darekar S, Klein G, Kashuba E. 2015. Different Mechanisms of Regulation of the Warburg Effect in Lymphoblastoid and Burkitt Lymphoma Cells. PLoS One 10(8):e0136142.

II. **Mushtaq M**, Gaza HV, Kashuba EV. 2016c. Role of the RB-Interacting Proteins in Stem Cell Biology. Adv Cancer Res 131:133-157. (Review).

III. **Mushtaq M**, Darekar S, Kashuba E. 2016b. DNA Tumor Viruses and Cell Metabolism. Oxid Med Cell Longev 2016:6468342. (Review).

IV. Kashuba E, **Mushtaq M**. 2017. Do MRPS18-2 and RB proteins cooperate to control cell stemness and differentiation, preventing cancer development? Exp Oncol 39(1):12-16. (Hypothesis).

CONTENTS

LIST OF ABBREVIATIONS

ATP	Adenosine tri phosphate
CDK	Cyclin dependent kinase
CEBPB	CCAAT/enhancer-binding protein
CENPF	Centromeric protein F
CRC	Colon adenocarcinoma
DAP3	Death associated protein 3
DCC	Deleted in colorectal carcinoma
DNMT1	DNA methyltransferase 1
DRYK1	Dual-specificity tyrosine phosphorylation regulated kinase 1
DS	Down Syndrome
E2F1	E2F transcription factor 1
EBV	Epstein-Barr virus
EBNA	EBV encoded nuclear antigen
EC	Endometrial cancer
EID1	CREBBP/EP300 inhibitory protein 1
EMT	Epithelial to mesenchymal transition
FADH2	Flavin adenine dinucleotide 2 (reduced)
HDAC	Histone deacetylase
hTERT	Telomerase reverse transcriptase in human
KDM5A	Lysine-specific demethylase 5A
LSU	39S large subunit of mitoribosome
MRP	Mitochondrial ribosomal protein
MYOD1	Myogenic differentiation antigen 1
NADH	Nicotinamide adenine dinucleotide
Nanog	Homeobox protein NANOG
NK	Natural killer
OCT4	Octamer-binding transcription factor 4
OXPHOS	Oxidative phosphorylation
PCa	Prostate cancer
PD	Parkinson disease
PELP1	Proline-, glutamic acid-, and leucine-rich protein 1
PHB	Prohibitin
PIK3CA	Gene encoding p110α protein
PSMD10	Proteasome 26S subunit, non-ATPase, 10
RB	Retinoblastoma associated protein
RB1	Retinoblastoma 1 gene
REF	Rat embryonic fibroblast
RNF2	Ring finger protein 2
RSFs	Rat skin fibroblast
RUNX2	Runt-related transcription factor 2
S18-2	Mitochondrial ribosomal protein S18-2
SIN3A	SIN3 transcription regulator family member A
SIRT1	Sirtuin 1
SKI	V-Ski avian sarcoma viral oncogene homolog
SMA	Smooth muscle actin
SOX	Sex determining region Y box
SPI1	Spleen focus forming virus proviral integration oncogene
SSEA	Stage-specific embryonic antigen
SSU	28S small subunit of mitoribosome
TCA	Tri-carboxylic acid

UHRF2	Ubiquitin-like protein containing PHD and ring finger domains 2
ZBTB7A	zinc finger- and BTB domain containing protein 7A

1 INTRODUCTION

1.1 MITOCHONDRIA

Mitochondria are present in all living organism from bacteria to plants and in humans. The origin of mitochondria is believed as a result of symbiosis more than 1.45 billion years ago. The earliest observations of mitochondria can be traced to 1840s [1]. However, in 1890 Richard Altmann realized the appearance of such cellular organelles [2]. He named them "bioblasts" and suggested that they were "elementary organisms" residing and performing pivotal roles in the cells. The name mitochondrion was introduced by Carl Benda in 1898 [3]. This name originates from the Greek words "mitos" (thread) and "chondros" (granule), due to their appearance during spermatogenesis.

Currently, two main theories explain the symbiotic derivation of mitochondria. They differ with regards to their speculation about the nature of host, the physiological capabilities of mitochondria and the type of ecological factors that were involved in successful symbiosis [4]. The traditional view postulates that mitochondrion was engulfed by an anaerobic nucleus-bearing eukaryotic host cell via phagocytosis. The competing theory posits that a prokaryote which was most probably an archaebacterium acquired the mitochondrion.

Mitochondria are diverse among species. In contrast to mammalian mitochondria, the mitochondria of certain invertebrates do not utilize oxygen. Such anaerobic mitochondria generate about 5 moles of adenosine triphosphate (ATP) per mole of glucose, contrary to 36 moles generated during aerobic respiration. Yet another type of mitochondria is found in protists eukaryotes, called hydrogenosomes which produce hydrogen as a major metabolic end product. Hydrogenosomes produce only 4 moles of ATP per glucose molecule [5]. The mitochondria of eukaryotic parasites like *Entamoeba histolytica* are called mitosomes, which do not produce any ATP molecules [4].

1.1.1 Mitochondrial structure and function

Mitochondria are diverse in structure and size, ranging between 0.75 to 3μm^2 across different species [6]. They are considered as autonomous cell organelles due to the following reasons: 1) they carry their own DNA which can replicate independently; 2) they possess their own ribosomes, namely, mitoribosomes; 3) mitochondria can synthesize some of their own structural proteins. The typical mammalian mitochondria have a double membrane structure whose lumen is filled with mitochondrial matrix [7]. The outer membrane of mitochondria is permeable to pyruvate [8], oxygen [9], ATP [10] and certain other molecules. The inner

membrane is intricated into many invaginated structures known as ‘‘cristae’’. The shape of the cristae is often flat or tubular but in some cases the number and shape of the cristae may vary. For example, in certain nerve cells, the cristae take the form of prisms [11] while in some photoreceptor cone cells they have a spiral shape [12]. The shape of the cristae is associated with the regulation of ATP synthesis in mitochondria [13]. The cristae are covered with many small stalked particles named inner membrane spheres. Each sphere is composed of a stalk and a sphere (head) [14]. The sphere is on the matrix side and it contains an enzyme called F1. This enzyme is involved in the production of ATP [15].

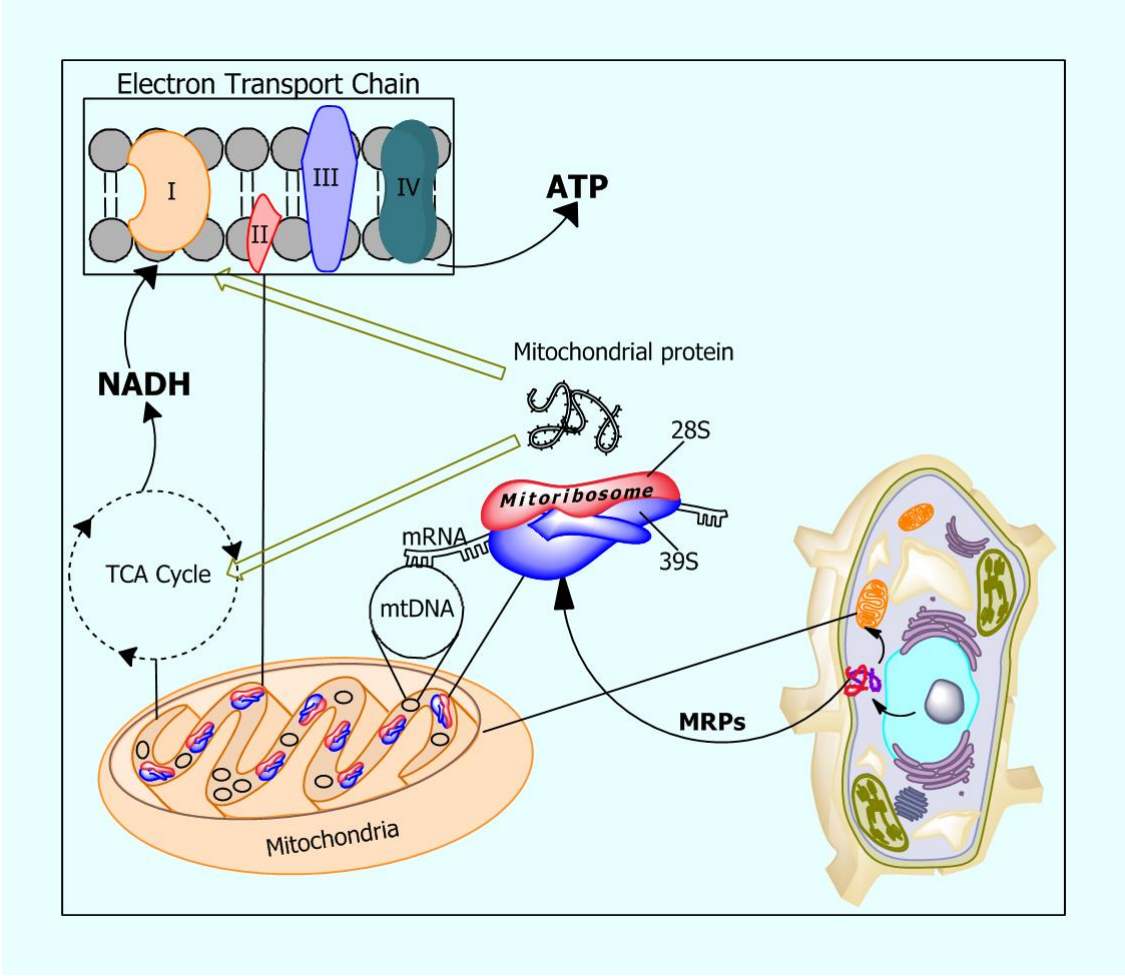

Figure 1: Mitoribosome. Each mitoribosome is comprised of SSU (red) and LSU (blue). The MRPs are encoded by genomic DNA in the nucleus, the product is processed in cytoplasm and then transported to mitochondria. Figure was drawn using Chem Biodraw software.

Mitochondria are also called power house of the cell because they fuel cells with energy in the form of ATP through the process of oxidative phosphorylation (OXPHOS). In the cytosol, glucose is catabolized to pyruvate in the process of glycolysis [16, 17]. Pyruvate transverses the outer membrane of mitochondria through porin (voltage dependent anion channel) [18]. In mitochondria, the pyruvate dehydrogenase complex system converts

pyruvate to acetyl-CoA [19] that take part in the tri-carboxylic acid (TCA) cycle (also termed as Kreb's cycle).

The TCA cycle is comprised of 10 biochemical reactions oxidizing two carbon atoms acetyl-CoA to carbon dioxide. The end product of the TCA cycle is the reduced form of Nicotinamide adenine dinucleotide (NADH) [20]. The products of TCA; NADH and FADH2 enter the electron transport chain (ETC), a sequential process of electron transfer between electron accepting and donating molecules that ends with ATP at the expense of oxygen. The ETC, which combines phosphorylation and oxidation, is composed of four multi-subunit complexes (Complex I-IV) along with ATP synthase (Complex V). The protein complexes involved in the ETC are encoded by both the mitochondrial and nuclear genome [21].

The five multimeric protein complexes of ETC are: NADH dehydrogenase, an ubiquinone oxidoreductase (complex I composed of about 46 subunits), succinate dehydrogenase another ubiquinone oxidoreductase (complex II containing four subunits), an ubiquinone cytochrome *c* oxidoreductase (complex III of 11 subunits), cytochrome *c* oxidase (complex IV comprised of 13 subunits), and ATP synthase (complex V, consist of 16 subunits). ATP synthesis is a two-step process; firstly, electrons derived from NADH and FADH2 are delivered to molecular oxygen through different mitochondrial complexes, producing water. In the next step protons are pumped through the inner membrane by complexes I, III, and IV, generating an electrochemical gradient [22].

1.1.2 Mitochondrial ribosome

In 1963, mitochondrial DNA (mtDNA) was discovered in the embryos of chicken, as fibers exhibiting the DNA like characteristics [23]. The DNA of mammalian mitochondria is circular [24] and is acquired by offspring solely from the mother [25, 26]. It was shown that after fertilization the paternal mtDNA of the sperm is tagged by ubiquitin and degraded in the embryo [27]. The mtDNA encodes 37 genes, including the 12S and 16S rRNAs, the 22 mitochondrial tRNAs and 13 essential peptides of OXPHOS system [28]. In human, a 14th biologically active protein was discovered, named humanin [29]. It is encoded by mtDNA; however, unlike other mitochondrial proteins, humanin does not localize in mitochondria. The 13 mRNA related to OXPHOS are translated to protein by mitoribosomes (Figure 1). In 1967 Thomas O'Brien and co-workers isolated the mitoribosomes from rat liver mitochondria [30].

During the evolution of mammalian mitoribosome (55S), the ancestral mitoribosome (70S) underwent key structural alterations. The 55S mitoribosome contains more proteins than the

bacterial ribosome; therefore, mammalian mitorobosomes are larger than bacterial ribosomes despite the loss of approximately half of their RNA [31].

Mammalian 55S mitoribosome is composed of two subunits: the 28S small subunit (SSU) and the 39S large subunit (LSU) [32-34]. The SSU is involved in mRNA binding [32] and decoding, whereas the LSU assists the mitoribosome in catalysis of peptidyl transferase reactions [35].

The mitochondrial ribosomal proteins (MRPs) are encoded by nuclear genomic DNA. Their mRNAs are transcribed in the nucleus, then MRPs are synthesized in cytoplasm before they are transported to the mitochondria (see Figure 1). Generally, the N terminus of MRPs is responsible for the translocation of these proteins to mitochondria [36-40]. Recently, the crystallographic structure of mammalian 55S mitoribosome was resolved at a resolution of 3.8Å, using cryo-electron microscopy [41].

1.1.3 Mitochondrial ribosomal proteins of small and large subunits

The LSU of mammalian mitoribosome carries 52 MRPs. It carries 11 extra proteins compared to the 54S LSU of yeast mitoribosome [35]. Some of the featured parts of LSU are peptidyl transferase center (PTC), the polypeptide exit site (PES), central protuberance (CP) and exit site (TE). The PTC is formed exclusively of rRNA and it catalyses peptide bond formation; MRPL59 may be involved in protein–RNA interactions in this region. At the CP, MRPL48 and MRPL40 may form a portion of the P-site finger. Other important MRPLs in CP region are MRPL38, MRPL18, MRPL40, MRPL46, MRPL48 and MRPL55. The nascent protein leaves the ribosome at PEC. In LSU, PES starts at the end of PTC and leads through the LSU to the polypeptide exit site. The PES region plays an important role in processing, folding and targeting of nascent polypeptides. In bacteria, this sequence is targeted by antibiotics [35].

The SSU of mitoribosomes contains 12S rRNA and 30 MRPs, 15 of which are specific to mammalian mitoribosomes. Fourteen have homologues in 30S ribosomal proteins of *E. coli* and more in *D. melanogaster*, *C. elegans*, and *S. cerevisiae* [32, 39]. The overall structure of SSU is elongated and is divided in three distinct regions; head, platform and foot [42] as shown in Figure 3. The head portion is recognized by presence of MRPS29 which is GTP binding protein, it was shown to be phosphorylated *in vivo* and is involved in inter sub unit bridge formation [43]. The foot carry MRPS27 which is penta-tricopeptide repeat (PPR) domain protein [44]. MRPS27 binds with tRNA (Glu) and 12S rRNA and these bindings are essential for translation, because MRPS27 knockdown causes deficiency in cytochrome c

oxidase activity and decreased abundance in respiratory complexes [45]. The mRNA entrance site in SSU is composed of MRPS5, MRPS24, and MRPS39. The diameter of the entrance site is constrained, shorter than the two strands of RNA therefore only single stranded mRNA (ssRNA) can pass. At the entry region, another PPR repeat protein (MRPS39) is located which binds mRNA molecules and direct them through the ribosomal channel. The exit site is mainly composed of MRPS28 and MRPS37. Unlike the bacterial homolog, mammalian MRPS28 strongly binds with ribosome through interactions with MRPS2 and MRPS21 [2, 44]. The SSU also display intrinsic GTPase activity [46] due to the presence of MRPS29 in the head, it binds with the 12S rRNA as well as MRPS7, MRPS9, and MRPS35. Such bindings result in inter-sub unit bridging between SSU and LSU [41].

1.1.4 S18 family of mitochondrial ribosomal proteins.

The MRPS18 (termed S18 here) proteins are grouped in a family, consisting of three homologs in metazoa (S18-1–3) and one homolog in other cellular organisms. S18-1 and S18-2 are localized on the SSU while S18-3 is present on LSU. Three S18 proteins remarkably differ in structure (Figure 2) and size, ranging from 11.7 to 27 kDa [28]. The gene sequences of *S18* are remarkably different among each other suggesting that they might perform different functions [47]. The sequence of S18 homologs are found to be more closely related to chloroplast S18 than to prokaryotic S18 [47]. The *S18-1* gene lies on chromosome 4q21.23 [48], whereas *S18-2* is located on chromosome 6p21.3.1 [48] (adjacent to the genes encoding the MHC class II proteins) and S18-3 was mapped to chromosome 6p21.3 [49].

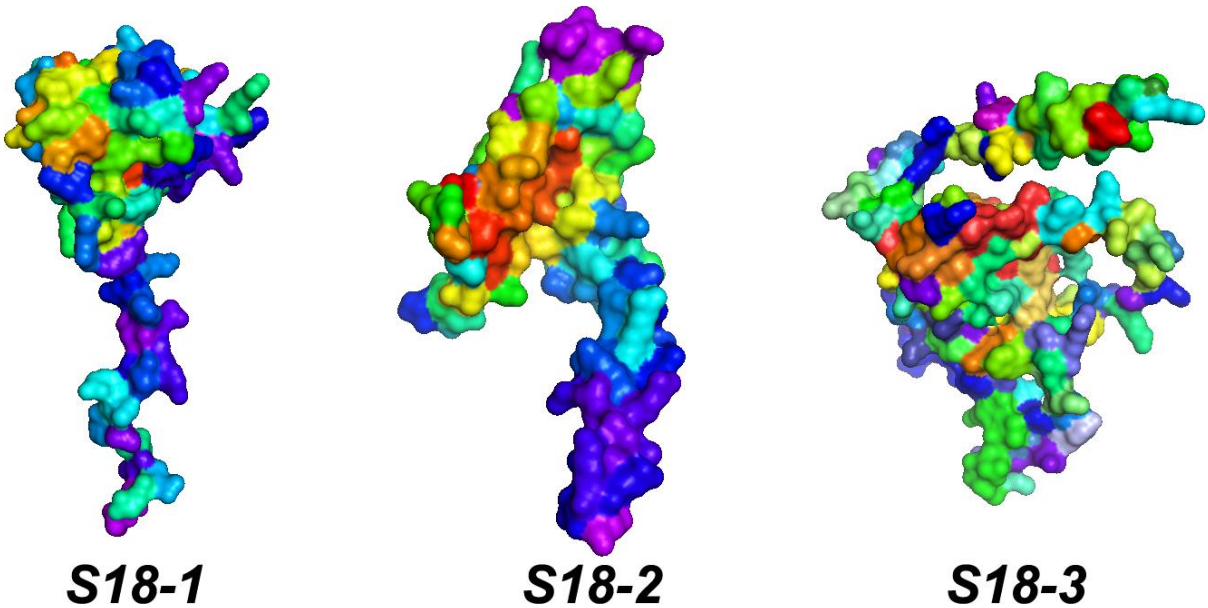

Figure 2: The crystal structure of S18 family of MRPs. Figure depicts that S18 proteins are structurally very distinct from each other. The structures were isolated using Pymol software from the entire 55S mitoribosome structure reported in [41].

Previously it was assumed that mitoribosomes are highly heterogeneous with each mitoribosome carrying only one of the S18 family proteins. [50] However, Greber *et al* showed that each S18 protein occupies a distinct position in mitoribosomes and that the three S18 proteins can be present in one mitoribosome [41]. The S18-1 binds to the same site as bacterial S18, while S18-2 occupies another site in the SSU. S18-3 localizes to the LSU and not to the SSU which was previously believed to be present on SSU [35, 51]. All three mitochondrial S18 homologs possess zinc-binding motifs. However, one of the zinc-coordinating cysteine residues is missing in both, S18-1 and S18-3. It is provided by MRPS6 and MRPL10 in trans, respectively. In case of S18-2, all zinc-binding cysteine residues are contributed by one protein chain, however only two of these residues form a typical CXXC-motif, while the third and fourth zinc-coordinating residues are distant in sequence. These unusual interactions involving two protein chains to coordinate the common zinc ion are probably needed for maintenance of quaternary structure and stabilization of MRPs. In mammalian mitoribosome, three of such interactions occur twice in case of S18 homologs, and one in MRPS25, where MRPS16 contributes the fourth zinc binding residue [35].

1.2 MITOCHONDRIAL RIBOSOMAL PROTEINS IN DISEASES

Recent advances have revealed an important aspect that some of the proteins from SSU appeared to be multifunctional and they perform extra-mitoribosomal functions. For example, the protein sequence of MRPS29 shows similarity with the death-associated protein 3 (DAP3), which is involved in apoptosis [52, 53]. It is not obvious whether its role as DAP3 is independent or employed as part of the ribosomal structure.

1.2.1 Developmental and neurodegenerative disorders

Differential expression pattern of MRPS6 has been shown in Down's syndrome (DS) and Parkinson’s disease (PS) mouse models. The DS is characterized by chr 21 trisomy [54]. Many clinical symptoms are associated with DS that differ in severity as well as prevalence among patients. Such symptoms include defects in neurogenesis, mental retardation, and also alterations in neuronal differentiation in the brain that results in earlier onset of Alzheimer's disease.

Expression profile of mouse ESCs carrying human chromosome 21 (hChr21) showed an altered expression of *S18-3* in the late differentiation of neurons [55]. The expression level of *Mrps6* was higher in mouse model of DS (Ts65Dn) [56]. The gene expression analysis of cerebral cortex, cerebellum and hippocampus of another DS mouse model Ts1Cje was

assessed. Ts1Cje harbors a triplicated chromosome 16, partially, homolog of human chromosome 21. The study reported that *Mrps6* was one of the genes with altered expression in brain [57].

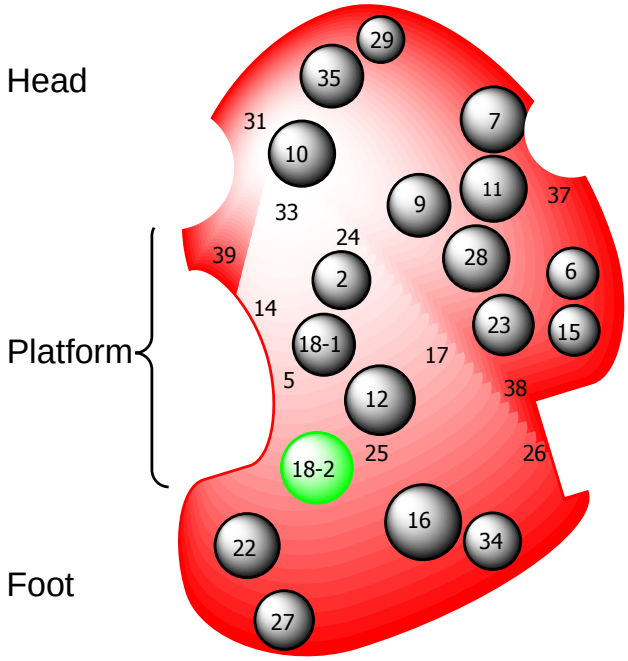

Figure 3: The localization of MRPs in three distinct regions of SSU. The MRPs in circle are reported in different diseases. S18-2 is denoted in green circle. Figure was drawn using Chem Biodraw software.

The differential expression of *MRPS6* along with other genes was found in PD, marking it a putative candidate for PD [58]. Similarly, array comparative genomic hybridization analysis indicated loss of *MRPS9* gene in sample isolated from a boy who showed intellectual disability and dysmorphic features [59].

1.2.2 Mitochondrial respiratory chain diseases

As mentioned above, the mtDNA encodes 13 proteins that are involved in the process of OXPHOS. Several mutations in genes encoded by mtDNA are associated with diseases, for example, the Leigh syndrome (mutation in genes of complex I); the cardiomayopathy Kearns-Sayre syndrome and hereditary paraganglioma (mutations in the genes encoding complex II); optic atrophy, hypertrophic, cerebellar ataxia and encephalomyopathies (mutation in the genes encoding Complex IV); Luft's disease (probably due to dysregulation of complex V) and undefined myopathies (due to a mutation in Coenzyme Q10) [60]. Mutations in the genes, encoding MRPs also cause severe respiratory chain dysfunction. A

homozygous nonsense mutation (Arg111Stop) in the MRPS16 led to a significant decrease in the activity of complex I and IV [61].

Mutation in the *MRPS22* gene was observed in case of Cornelia de Lange-like disease. The genetic cause of this disease is substitution mutation at Leu215Pro in the MRPS22 protein sequence that was detected in patient fibroblasts. Importantly, transfection of those cells with normal *MRPS22* cDNA reverted the phenotype [62]. Another missense mutation (Arg170His) in the MRPS22 protein sequence was found in patient with mitochondrial dysfunction. Subsequent transfection of the patient cells with wild-type *MRPS22* cDNA increased the 12s rRNA transcript level and normalized the enzymatic activities [63]. Also, in case of lactic acidosis the homozygous mutation of *MRPS22* gene was found [64].

Functional analysis of patient fibroblasts carrying mutated MRPS16 or MRPS22, was performed to find their impact on the integrity of SSU and LSU. Noteworthy, the low expression of MRPS16 also affected the levels of MRPS11 with inappropriate assembly of mitoribosome. However, the levels of MRPS16 had no effect on the LSU, limiting the effect to the SSU of mitoribosome. The retrieved mitoribosomes that harbor mutation (Arg170His) in the MRPS22 protein sequence contained less MRPS16 and MRPS11 [65]. In respiratory chain dysfunction other mutation, namely (Met184Val) in the MRPS7 protein sequence was implicated [66]. Mouse carrying a mutation in *Mrps34* gene showed similar characteristics [67].

1.2.3 Cardiovascular disease

Mitochondria supply more than 90% of ATP required for the physiological function of heart and they play an important role during ischemia/reperfusion injury.

The polymorphism of *KCNE2* and *SLC5A3/MRPS6* genes was found to be associated with high mortality of myocardial infarction in the Polish population [68]. The same single nucleotide polymorphisms (SNP) of the *MRPS6* loci along with other 8 loci were identified in a large cohort study [69]. Mutation of *Mrps33* was found in a minute syndrome of *D. melanogaster* [70]. MRPS10 appeared as one of the three crucial factors for better response to β-blocker therapy [71]. To identify the loci responsible for a high blood pressure susceptibility, genome wide analysis was carried out in obese adolescents, revealing association of *MRPS22* loci with the high blood pressure [72].

1.2.4 Cancer

The overall theme of this doctoral thesis is to study the role of S18-2 in cancer. Apart from previous findings of our laboratory, others have demonstrated that S18-2 is expressed at significantly higher levels in tumor stroma and stroma of recurrent breast cancer [73] and other types of cancer as shown in Figure 4. The S18-3 was also expressed at high levels in breast cancer in a study carried out by selecting phage antibody libraries on breast tissue sections [74].

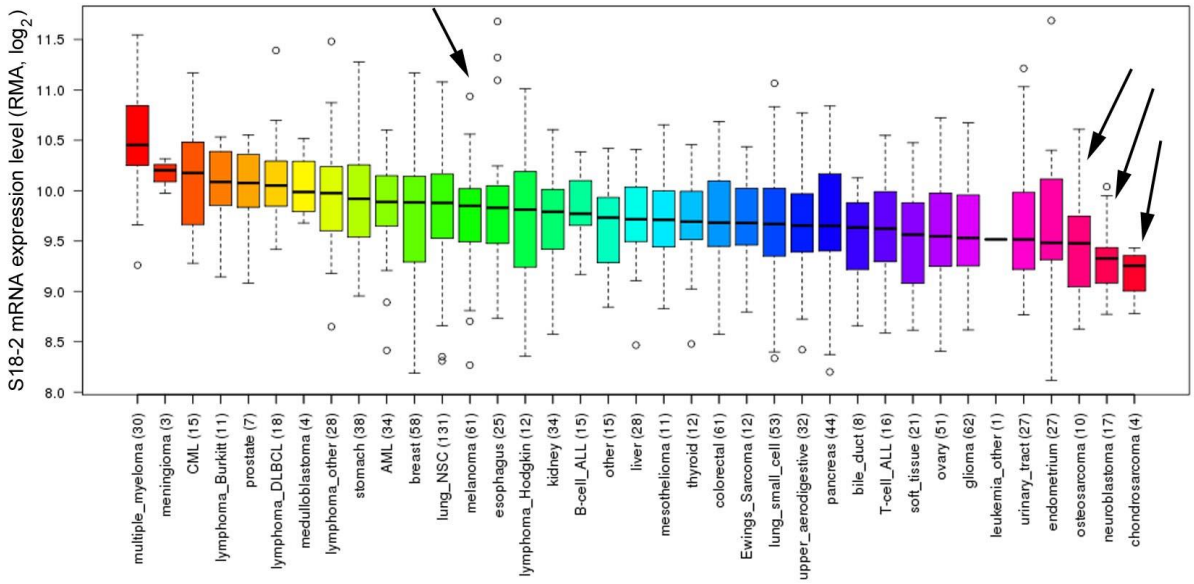

Figure 4: The mRNA expression levels of S18-2 in different cancers. Figure was adopted from [75].

The association of defective versions of several MRPs have been reported with increased risk of cancer development (Table 1). For instance, amplification of *MRPS23* gene was associated with increased proliferation in luminal type of breast cancer [76]. While, SNP of *MRPS30* genomic region were identified to be correlated with increased risk of postmenopausal breast cancer [77-81].

1.3 AN OVERVIEW OF CANCER

Tumorigenesis is a multistep process encompassing genetic modification that drive the successful transformation of normal cells into aggressive malignant tumor cells [82]. The genetic and epigenetic changes that are commonly associated with most of the cancer types may be grouped into ten essential characteristics, designated as hallmarks of cancer. Such traits include independency on external growth signals, avoiding growth inhibitory signals, escaping apoptosis, immortal replicative potential, angiogenesis, metastasis and tissue invasion, immune evasion, metabolic dysregulation, tumor-promoting inflammation and

chromosomal instability [83, 84]. When these hallmarks are acquired as physiological capabilities, they collectively dictate malignant growth. The hallmarks of cancer may provide a guideline for future therapies targeting cancer [85]. In this doctoral thesis, I describe in detail the involvement of S18-2 in at least four features of cancer - immortalization, metastasis, genomic instability and cellular differentiation (see Figure 6).

1.3.1 Oncogenes and Tumor suppressor genes

Cancer cells differ from their normal counterpart in many physiological aspects, such as morphology, gene expression pattern and intercellular interactions, growth control and structure of cytoskeleton. With respect to gene expression two major classes of gene play important role in cancerogenesis - the oncogenes and the tumor suppressor genes (TSGs). The corresponding proteins regulate cell growth and proliferation; mutations and aberrant expressions of such genes induce tumorigenesis. Most of the cancer harbour mutations, deletions or promoter methylation of TSGs that leads to inactivation of TSGs, controlling cell cycle. On the other hand, gain of function by mutations or chromosomal rearrangement make oncoproteins constitutively active. Proto-oncogenes were discovered in carcinogenic retroviruses. The oncoproteins deliver excessive and uncontrolled growth promoting signals to cancer cells; few examples of well-known proto-oncogenes are *Myc*, *Ras* and *Src*. A striking difference between oncogenes and TSGs is that oncogenes act dominantly while the TSGs operate in recessive manner i.e. both alleles of the TSG must be mutated, deleted or inactivated to induce cancer. Retinoblastoma susceptibility (*RB1*) gene, *p16* and *p53* represent TSGs that are inactivated in many tumor types [86].

1.3.2 Cell immortalization

Normal human cells are endowed with an ability to replicate only a certain number of times. Normal cell populations cease proliferation when they have exhausted their allotment of permitted doublings and even enter apoptosis [87]. However, such limited proliferation conflicts with fundamental characteristics of cancer cells. During the period of development of neoplastic growth, human cells go through multiple successive growth and division cycles. Provided with proper nutrients, these cells may show unlimited replicative potential in culture and are thus said to be immortal. It is estimated that the adult human body comprises of approximately 3.72×10^{13} cells [88] and the organism as a whole undergoes at least 10^{16} cell division during its life-time [89]. Normal cells in general undergo approximately 50 population doubling, what separates them from multipotent cells in early embryogenesis.

Table 1. The differential expression of *MRPs* genes in cancer. The data was adopted from [42] where authors in turn extracted information from the Oncomine database.

Cancers	Upregulated	Downregulated
Bladder cancer	MRPS2, MRPS7, MRPS12, MRPS15, MRPS17	
Brain and CNS cancer	MRPL42, MRPS28, DAP3	
Breast	MRPS7, MRPS10, MRPS12, MRPS14, MRPS17, MRPS21, DAP3, MRPS30, MRPS34	MRPS5, MRPS6, MRPS15, MRPS23, MRPS27
Cervical	MRPL42, MRPS6, MRPS11, MRPS15, DAP3	
Lung	MRPL42, MRPS12, MRPS17, MRPS26, MRPS30, MRPS33	MRPS15
Esophageal	MRPS6	MRPS18-1, MRPS10, MRPS22
Gastric	MRPS10, MRPS12, MRPS17, MRPS26, MRPS28, DAP3, MRPS30.	
Head and neck	MRPS7, MRPS9, MRPS12, MRPS14, MRPS15, MRPS17, MRPS23, DAP3, MRPS30	MRPS2
Kidney	DAP3	MRPS6
Colorectal	MRPS2, MRPS10, MRPS12, MRPS15, MRPS17, MRPS23, MRPS27, MRPS30, MRPS31	MRPS5, MRPS6
Leukaemia	MRPS12, MRPS23, MRPS27, DAP3, MRPS30	MRPS 25, MRPS31
Liver	MRPL42, MRPS17, MRPS21, DAP3	
Lymphoma	MRPS7, MRPS9, MRPS11, MRPS12, MRPS14, MRPS15, MRPS 17, MRPS18-3, MRPS18-2, MRPS21, MRPS24, MRPS25, MRPS26, MRPS28, MRPS29, MRPS31, MRPS33, MRPS34, MRPS35, MRPS36	MRPS6, MRPS30, MRPS31

Leonard Hayflick demonstrated in the 1960s that cultured cells isolated from human or rodents stopped further growth after certain number of divisions. Those cells enter into a state of so called replicative senescence, once achieving the expected number of replicative growth

cycle [90, 91]. Senescent cells might be metabolically active but they lose their ability of replication [92].

During the G_2 phase of the cell cycle, ends of the long arms of two sister chromatids are fused together. Once the cell cycle enters the M phase (interphase), the spindle fiber binds to the centromere and pulls apart the two sister chromatids in opposite directions, unaware that the chromatids are joined at the ends of long arms [93]. The end to end fused arms should be separated cleanly. Otherwise the chromosome breakage could occur at weak points of chromatids [94].

Telomeres are small tandem repeats of hexanucleotide sequence (5-TTAGGG-3) [95] present at the end of chromosome [96]. In immortalized and transformed cells the telomeres are continuously added to the end of chromosome by telomerase (hTERT) [97]. The hTERT is a complex of two subunits; a catalytic holoenzyme and the RNA. The catalytic subunit has reverse transcriptase activity [98] and uses its accompanying RNA subunit [99] as a template to synthesize the telomeric DNA. The length of telomeres determines the number of division of a cell. In normal cells after each replicative cycle telomere is shortened in the length, it becomes too short after certain number of cell cycles that it is unable to protect the chromosome anymore [100]. In that case the cells undergo crisis and consequently dies [101].

1.3.3 Migration and metastasis

The spreading process of cancer is termed ‘‘metastasis’’ and is the major cause of tumor progression and patient’s death [102]. During metastasis the tumor cells behave autonomously [103] and do not respect their usual boundaries; therefore, cells may migrate and invade other parts of the body. Metastasis can be local when the tumor spreads to another area of the same organ or it could be distant. In the latter case the malignant cells enter the blood flow or lymphatic system and reside to other tissues or organs of the body. In order to gain the migratory and invasive ability carcinoma cells must undergo the process of epithelial to mesenchymal transition (EMT) to shed most of their epithelial phenotype [104]. During this process tumor cells growth pattern resembles to wound healing mechanism where the same phenomenon of EMT occurs. Under embryogenesis, during one stage of gastrulation the cells from ectoderm also migrate inside towards the centre to form mesoderm [105]. EMT is characterized by loss of expression of the epithelial markers E-cadherin [106, 107] and certain cytokeratins, and the gain of mesenchymal marker expression like vimentin, N-cadherin and fibronectin [108]. E-Cadherin enables epithelial cells to attach one another by forming homodimeric bridges between adjacent cells. The ectodomain of E-cadherin extends

from plasma membrane of one epithelial cell and make a complex with other E- cadherin molecule, protruding from the surface of an adjacent cell [109]. Once the tumor cells arrive to new site, they regain their epithelial phenotype by reversing the EMT through the mechanism of mesenchymal to epithelial transition (MET) [110].

1.3.4 Cell stemness and differentiation

Stem cells (SCs) are pluripotent cells that exhibit two distinctive characteristics: first the ability to maintain self-renewal and second the potential of differentiation into specialized cell lineages. In mammals, normally two types of SCs are identified. The first type is represented by the pluripotent embryonic stem cells (ESCs), existing in inner cell mass of blastocyst which can differentiate into any kind of somatic cells. The second type of SCs are adult stem cells that are reside in most of tissues and are believed as a part of repair system to replace damaged cells of the tissues. Highly organized and regulated signaling systems control the two fundamental characteristics of SCs, such as self-renewal and differentitation. Under normal physiologic conditions, controlled shifts in balance of such signaling pathways induce differentiation. Abnormalities in signaling cascades can initiate and promote cellular transformation and oncogenesis [111]. Apart from normal cells, cancer cells are also believed to arise from a population of cells that show SCs like properties called cancer stem cells (CSCs) [112]. Concept of CSC in maintenance of cancer cells was put forwarded few decades ago and has attracted great interest but remains controversial [113].

Induced pluripotent stem cells (iPSC) are also a type of SCs that are generated in vitro from reprograming of differentiated adult cells. The first iPSCs were produced in 2006 by introduction of four transcription factors namely *C-Myc Oct4, Nanog* and *Sox2* [114]. Several other genes have been identified that are associated with pluripotency of SCs, such as *Klf4, Nac1, Essrb, Sall4, Tcl1, Dax1, Tbx3, Zfp281* and *Rif1* [115-117]. Proteins encoded by pluripotency associated genes modulate the activity of each other to form transcriptionally regulated complex networks in ESCs [118].

The SCs divide in asymmetric manner giving rise to two progeny cells with different cellular fates. One of the daughter cells retains its identity as SC, other proceeds to spawn a population of transit amplifying cells that terminally differentiate to mature cells. It has been proposed that during the asymmetric division of SCs, the daughter cell that remain in SCs compartment receive both the parental DNA strands while the newly synthesized DNA strand are allocated to the other daughter cell who is destined for differentiation [119]. It is believed that SCs adopt such strategy to protect its genome from genetic damage. Each time cell

passes through S phase, DNA polymerase makes mistake during the replication that escape from repair mechanism. Consequently, the parental strand that are not synthesized during the most recent cycle of DNA synthesis are more likely to preserve wild type sequences than are those strands that are the product of recent DNA replication [119].

In this thesis, prostate, endometrial and colon cancer are used as models to show the status of S18-2 in tumors.

1.3.5 Prostate cancer

Prostate cancer (PCa) is the most frequently diagnosed cancer in men. According to Swedish national board of health and welfare database (Socialstyrelsen), 10 440 men were diagnosed with PCa and 2 357 PCa related deaths were recorded in Sweden in 2015 (Available at: http://www.socialstyrelsen.se/statistik/statistikefteramne/cancer).

The development of prostate starts as an outgrowth of epithelial buds in the urogenital sinus in late embryogenesis (embryonic day 17.5 in mice) [120] These buds rapidly extend and undergo morphogenesis of branching under the influence of androgen, resulting in the formation of a glandular architecture of prostate. The initial organ outgrowth continues vigorously after birth and it is largely completed before reaching sexual maturity. In contrast, the gland becomes mainly quiescent in adult, with very few cells displaying either mitotic cell divisions or apoptotic cell death [121]. The male testosterone hormone plays an important role during prostate initiation [122]. In adult prostate, the loss of androgen signaling results in rapid death of the majority of luminal epithelial cells, which express high levels of androgen receptor and require androgen signaling for their morphogenetic development, survival and maintenance [123, 124].

Prostate cancer is the malignancy of prostate epithelial cells. Since 1986, early diagnosis and management of PCa has been developed, when tests for measuring Prostate-specific antigen (PSA) levels in serum were introduced into clinical routines. The PSA protein is produced by normal prostate cells at low levels. PSA levels in patient's serum increases with enlargement of prostate, that may be a sign of prostate cancer [125]. However, similar changes in the PSA levels are also characteristic for patients with benign prostatic hyperplasia [126] and with acute bacterial prostatitis.

In 2012, it was suggested that PCa screening based on PSA levels should not be used due to high rates of false-positive [127]. Despite this, one-third of men aged 65 years and older were screened in 2013 for PSA levels [128].

Most PCa–related deaths are due to the advanced metastatic stage of the disease that can spread locally or distantly to lymph nodes and bones. The mechanisms underlying PCa metastasis are incompletely understood both at the cellular and molecular levels [129].

Gleason system evaluates the PCa growth pattern based on the microscopic analysis of surgical section of prostate specimen. The more PCa tissue differs in morphology from normal prostate tissue, the more severe is cancer. The prostate tissue samples are graded from 3 to 5, with 3 as the least serious and 5 as the most serious degree of PCa [130].

The severity of PCa can be reduced using hormone therapy. The PCa cells even those that metastasized beyond the prostate gland need testosterone to grow. Therefore, the severity of PCa at all stages can be reduced by preventing the production of testosterone in body. Hormone therapy involves different strategies for blocking of testosterone synthesis like; surgical castration, medical castration (inhibiting testosterone production through drugs), administration of estrogen and anti-androgenic drugs. If patients are unresponsive to hormonal therapy then other procedures are applied such as chemotherapy, radiation and isotope treatment.

1.3.6 Endometrial cancer

Endometrial (uterine) cancer is initiated in the layer of cells that form the lining (endometrium) of the uterus. Between 2005 to 2015, each year about 1400 new cases of EC were reported in Sweden (Socialstyrelsen). The disease is rare before the age of 40 and older women are primarily affected by EC.

The estrogen and progesterone are produced by ovaries. Normally, these two hormones are in balance to each other. However, an imbalance towards more estrogen increases a woman's risk for developing EC. The factors that influence hormonal levels include taking estrogen, tamoxifen and birth control pills, the number of menstrual cycles (over a lifetime), pregnancy and a number of births, obesity and also polycystic ovarian syndrome. This increases the risk of EC occurrence. Other risk factors are diabetes, high fat diet, age, family history and other type of cancer.

The signs and symptoms of EC involve unusual vaginal bleeding, spotting and pelvic pain with weight loss. The diagnosis of EC is based on microscope examination which is possible only when tissue sections are isolated from patients. Currently, two systems are used for EC surgical staging - International federation of gynecology and obstetrics (FIGO) and American joint committee on cancer (AJCC). Both these systems are based on the TNM: tumor extent

(T), spread to lymph node (N) and distant sites (M). Usually surgery, chemotherapy, hormonal and radio-therapy are used to treat women with EC.

There are two types of EC. The type 1 is morphologically classified as endometrioid carcinoma. The type 1 accounts for 80% of all the EC cases and affects both pre- and post-menopausal women. Type 2 is morphologically clear cell or serous carcinoma and affects postmenopausal older women, as a rule. Type 2 are often poorly differentiated and have a worse prognosis, it develops directly from the endometrium without occurrence of hyperplasia. Type 1 is characterized by several genetic changes, but mutations in the tumor suppressor gene *PTEN* is most frequently observed. The mutations in *PTEN* are observed in up to 55% of precancerous endometrial lesions and 83% of EC [131, 132].

1.3.7 Colon cancer

Colon adenocarcinoma (CRC) is the most common type of gastrointestinal cancer. Colorectal cancer is the third most common cancer after breast and prostate cancer in the world. In Sweden, 4 490 new cases of colon cancer were detected, including 2 222 men and 2 268 women during 2015 (Socialstyrelsen). The statistics indicates that the incidence of CRC is equal in men and women. The risk of CRC increases with age. The etiology of CRC includes many factors, such as genetic and epigenetic alterations, environmental exposures (including diet) and inflammation of digestive tract. The common clinical symptoms associated with CRC include rectal bleeding, iron-deficiency anemia, abdominal pain and intestinal obstruction or perforation. In 2006, it was decided to introduce an organized CRC screening program in Sweden (Stockholm) [133]. The Council of the European Union issued recommendations in 2003 for FOBT based CRC screening in men and women aged 50–74 [134]. Colon cancer is often detected during screening; other procedures of diagnosis are colonoscopy, sigmoidoscopy, biopsy of suspicious lesions and double-contrast barium enema.

A genetic model for colorectal tumorigenesis called ‘‘Vogelgram’’ was proposed in 1990 by Eric Fearon and Bert Vogelstein. The model describes key genetic and epigenetic events at different stages of colorectal carcinoma, namely loss of chromosome 5q, 12p, 18q and 17p, and mutations of *APC, KRAS, DCC,* and *p53* genes that were mapped in sequential order of cancer progression [135]. Later on, others found that mutation in *BRAF*, *KRAS*, *PIK3CA* genes and loss of heterozygosity of chromosome 18q are commonly associated with CRC incidence. Several studies identified genetic and epigenetic biomarkers in plasma for CRC detection. The overall specificity of such biomarkers up to 96% with sensitivity ranges

from 30% to 87%. [136]. The CRC are also divided in different stages on the basis of TNM classification.

RB PROTEIN AND ITS ROLE IN CANCEROGENESIS

1.3.8 Cell cycle and RB

Most normal cell in body need external signals, such as those transmitted by mitogenic growth factors before they will decide to grow and proliferate [137]. Only early embryonic cells are exempt to this rule; they are able to multiply without receiving growth-stimulating signals. Since normal cells take part in the formation of precisely structured tissues their proliferation must be coordinated with adjacent cells in tissues. The body cannot give permission to each of its almost 10^{14} cells to decide on its own to divide and grow, such scenario might create chaos [89]. Therefore, in a tissue the fate of normal cells is governed through cell cycle clock which make decision based on the wide variety of incoming signals. The cell cycle that operates in the nucleus of cells includes mechanisms that control the timing and rate of DNA duplication with cell division.

The period between successive divisions of a cell is defined as the cell cycle, contents of the cell must be accurately replicated during this period. In 1950s, Alma Howard and Stephen Pelc observed in the broad bean, (*Vicia faba)* that DNA replication took place only at a specific phase of the cell cycle and that this stage was clearly separated from mitosis [138, 139]. Based on these observations, other investigators identified the four characteristic phases of the cell cycle: G_1, S, G_2 and M phase [140, 141]. The DNA replication is deferred for approximately 12-15 hours between the birth of new daughter cells and subsequent DNA synthesis. This period of cell cycle is called G_1 (first gap) [89, 142]. The decision about growth and quiescence is made by cells during a specific period in the G_1 phase [143]. Evidences indicate that cells consult their growth-regulating signals during a distinct window of time in the active cell cycle: from the onset of G_1 phase through most of G_1, ending an hour or two i.e. 80-90% of G_1 phase [144]. The cultured cells failed to precede the cell cycle when external growth promoting factors are removed before they reached the decision making 80-90% of G_1 phase [143]. However, once the cells passed through the restriction point (termed R point) and advanced into the final portion of G_1 (last 10-20% of G_1) the elimination of growth promoting factors has no longer effect on their progress [137]. Then they can proceed through the remaining G_1 and thereafter complete all the phases of cell cycle [89, 143].

The G_1 phase is followed by DNA synthesis phase termed as S-phase of the cell cycle that often requires 6 to 8 hours to reach completion in many mammalian cultured cells [89, 145]. The actual length of S phase varies greatly among different type of cells; it might be much shorter in rapidly dividing embryonic cells and lymphocytes.

After S phase, a cell might enter directly to mitosis (M phase). However, most mammalian cells postpone their entrance into M phase, staying 3 to 5 hours in a second gap period, named G_2 phase before they proceed to M stage [89, 145]. During G_2, the cell prepares itself for admission into M phase and cell division. The M phase itself takes about an hour and it includes four distinct subphases of karyokinesis; prophase, metaphase, anaphase and telophase [89, 145]. An overview of cell cycle is graphically represented in Figure 5.

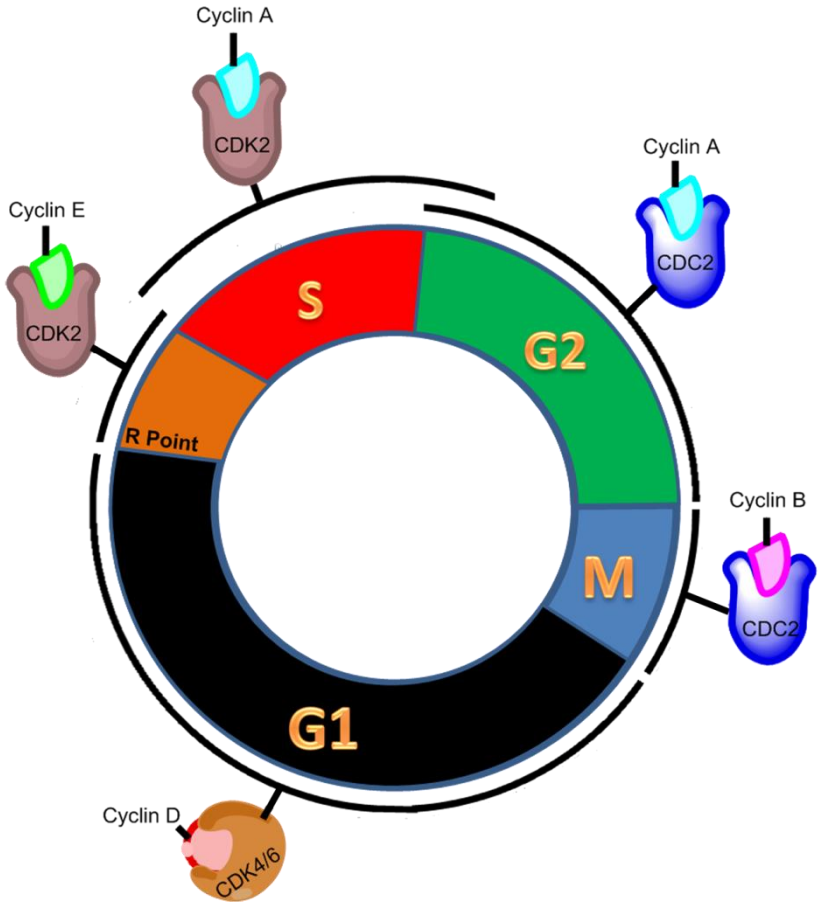

Figure 5: Representation of four stages of cell cycle. The G_1 black, orange the G_1 phase after R point, S phase red, G_2 phase green and M phase in blue color. The corresponding CDKs in complex with cyclin are shown. Figure was drawn using Chem Biodraw.

The explanation of how R point transition is precisely executed remained unclear until the first tumor suppressor gene, the retinoblastoma susceptibility (*RB1*) gene was identified and isolated by positional cloning during the mid-1980s [146-149]. Alteration in *RB1* gene was found to be associated with pathogenesis of retinoblastoma, sarcomas [146], glioblastomas

[150], small-cell lung [151], bladder [152], breast [153], cervical [154] and parathyroid cancers [155], supporting the idea that the gene and the translated protein is involved in a tumor suppressing pathway. The human retinoblastoma gene *RB1* is a prototype of tumor suppressor genes [156]. The cloning of the *RB1* gene and determination of homozygous *RB1* mutations in retinoblastoma follow the Knudson (1971) model, which states that a tumor phenotype appears only when both copies of the gene are altered [157]. The heritable form of retinoblastoma is caused by deletion of *RB1* usually, in that case the appearance of retinal tumors occurs with an incidence of more than 95% [158]. Importantly, the retinoblastoma is mostly a childhood cancer. The cause of retinoblastoma in a specific age period indicates a model of tumor initiation in which loss of RB function must happen in certain populations of cells that may be transiently exist in the retina of developing eye [159].

1.3.9 Gene and protein family of RB

The *RB1* gene is comprised of 27 exons spanning approximately 180-kb of genomic DNA on human chromosome 13q14. The locus transcribes a 4.7-kb transcript that encodes a nuclear phosphoprotein consisting of 928 amino acids, the retinoblastoma protein (RB/p105) [160]. Two RB related proteins also exist, the p107 and RB2/p130 that are located on human chromosome 20q11.2 and 16p12.2, respectively [157].

During cell transformation, viral oncoproteins target the RB family proteins [161, 162]. The sequences specific for binding to oncoproteins are called “pocket” which is common among three RB proteins, hence these proteins are often termed pocket proteins. Structurally, the pocket domain contains two conserved functional domains (A and B) that are separated by a spacer (S) sequence. The spacer is substantially different between the three RB proteins. The presence of a motif with LxCxE sequence is usually characteristic feature of proteins that target RB family proteins. A tridimensional LxCxE-binding site is located in the B-domain of pocket region of RB proteins. Several RB targeting viral oncoproteins have been identified, for example, early-region 1A (E1A) of the human adenovirus that carry the LxCxE motif [163]. The E7 protein from a high-risk human papilloma virus consists of three functional domains: the CR1 and CR2 in the N-terminus, and a large zinc-finger domain in the C-terminus. The CR2 sequence contains the LxCxE motif for binding to RB pocket proteins [164]. The large T antigen of polyomaviruses like simian virus 40, JC and BK viruses also possess LxCxE motif that target the RB proteins [165]. Herpes viruses developed more sophisticate way to target the RB-E2F pathway. For example, EBV-encoded nuclear antigen 6 (EBNA-6) inactivates RB by binding with the S18-2 protein, that serves as a bridge between EBNA-6 and RB [166]. The consequence of such binding is the inactivation of RB

proteins and transactivation of responsive genes, required for inducing the progression of cell cycle [163]. The targeting of RB protein by viral oncoprotein paved the way for the understanding of TSGs.

The RB2/p130 and p107 are closely resembled each other, sharing about 50% amino-acid sequence identity, compared to RB/p105 (approximately 30-35% identity). Noteworthy, mutations in *p130* and *p107* are not common in human cancers [167]

1.3.10 Mechanism of action of RB

The retinoblastoma protein is broadly studied for its role in the regulation of cell cycle. RB directly binds the transcription factor (TF) E2F1. This binding leads to inhibition of transcriptional activity of E2F1, and it remains inactive during the G_0 and M phase of cell cycle [137]. Phosphorylated state of RB cannot interact with E2F1 but RB binds and inactivates E2F1 when it is unphosphorylated. RB is mostly unphosphorylated when the cells are in G_0 phase. It becomes weakly phosphorylated (hypophosphorylated) at specific serine and threonine residues during the first 80-90% of G_1 phase, while it is extensively phosphorylated (hyperphosphorylated) at R point gate, thereafter, RB remains hyperphosphorylated throughout the cell cycle [157, 168].

The phosphorylation of RB is controlled through cyclin dependent kinases (CDKs) [169]. The CDK4 and CDK6 are growth signal dependent CDKs that operate during first 80-90% of G_1 phase while growth signal independent CDKs (CDK1, 2 and 3) are active in the rest of cell cycle [89] [170]. CDKs need cyclin molecules (Cyclin D, E and A) as a guide for phosphorylation of RB according to the requirement of cell cycle at different stages [171] [172]. Apart from cyclins, cells also employ CDKs inhibitors to regulate the activity of CDKs and thus inhibit phosphorylation of RB [171, 173]. CDK inhibitors are classified into two categories; p16INK4A, p15INK4B p18INK4C and p19INK4D inhibitors of CDK4/6 [174] and p21Cip1, p27Kip1 and p57 Kip2 for inhibition of CDK1, 2 and 3[175].

The inactivation of RB led to uncontrolled cell division that might result in development of cancer. Cancer cells adopt different strategies for inactivation of RB such as mutation and/or deletion of *RB*1 gene, epigenetic silencing of *RB1* gene expression, inactivation of CDK inhibitors, mutation in *CDK* genes and recruitment of certain proteins that binds RB and prevent its association to E2F1 [89].

1.3.11 RB binding partners

As mentioned above that oncoproteins target the RB protein to drive the cellular transformation. The literature review reveals that RB binds to 218 proteins; we have summarized the data for 27 of them which are important in cell stemness or cancer development. Apart from RB control over cell cycle through E2F1, RB can bind to other proteins which are indirectly involved in cell cycle like LIN9, DYRK1A, and S18-2 protein. Some RB-interacting proteins like ZBTB7A [176], SKI [177], EID1 [178], LDB1 [179] and PSMD10 [180] are involved in maintenance of pluripotency and self-renewal of SCs. HBP1 [181], SPI1 [182], CEBPB [183], MYOD1 [184], CENPF [185], PELP1 [186] are also RB associated proteins that regulates lineage specific differentiation of cells. Yet other RB binding proteins like SIN3A [177] control proliferation through regulating the *c-Myc* level [187], TRAP1and PHB protect cells from apoptosis [188]. Many RB interacting proteins are involved in epigenetic regulation of gene expression for example; UHRF2 (DNA methylation) [189], HDAC (Histone deacetylase) [190], KDM5A (H3K4 demethylation) [191], SIRT1 (H4K16 acetylation) [192] and DNMT1 (DNA methylation) [193].

1.3.12 S18-2 and RB

The S18-2 was initially cloned from hematopoietic SCs [194]. Working with Epstein-Bar virus (EBV), it was found in our laboratory that S18-2 formed a bridge between RB and EBV encoded nuclear antigen 6 (EBNA6). Evidences based on the results of yeast two hybrid system, GST pull down and surface plasmon resonance showed that S18-2 binds RB. The RB association to E2F1 was inhibited due to such binding, as the free E2F1 levels significantly increased in the nucleus of S18-2 overexpressed cells [166].

The binding site of S18-2 in RB is not known yet but most probably it is present either in C-terminus or middle region of RB protein, as S18-2 binding with RB was observed when a RB construct was used with deleted N-terminus. As mentioned above that overexpression of S18-2 led to immortalization of rat embryonic fibroblasts (REFs). The S18-2 immortalized cells induced the expression of embryonic SCs markers like SOX2, OCT4, SSEA1, Nanog etc. [195].

The gene expression analysis showed that more than 2000 genes were differential regulated with overexpression of S18-2 compared to primary REF. The upregulated genes were those involved in the transcription/translational machinery of redox reaction like elongation factors, ATP synthases, mitogen activated kinases, NADH dehydrogenases. The genes were involved in pathways which are characteristics of rapidly proliferating cells like P13K/AKT signalling,

pathways involved in the OXPHOS, ubiquinone (coenzyme Q10) biosynthesis and fatty acid

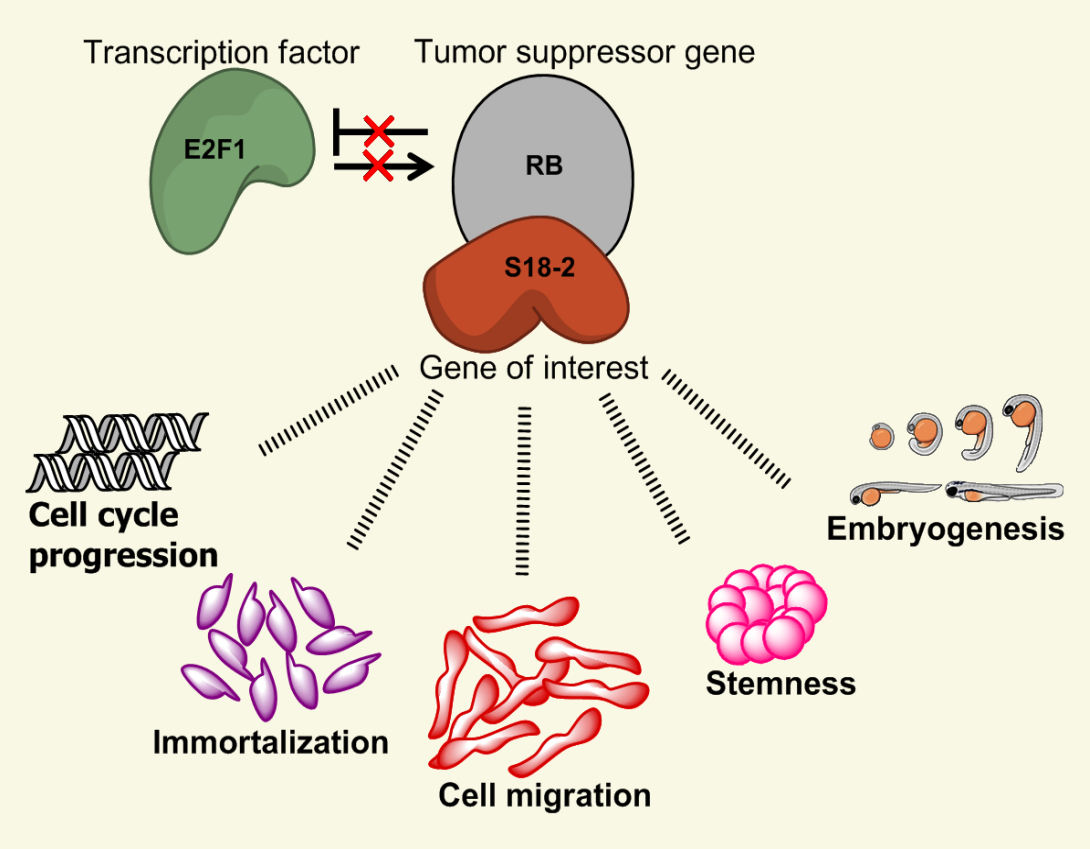

Figure 6: S18-2 and RB interaction. The graphical abstract of study. Figure shows that S18-2 binds to RB resulting in RB-E2F1 association. The study describes the effect of RB-S18-2 in embryogenesis, stemness, cell migration, immortalization and advancement of cell cycle.

elongation in mitochondria were upregulated. Cells immortalized by overexpression of S18-2 produced more pyruvate, indicating enhanced ATP synthesis [196].

2 AIMS OF THE STUDY

The overall aim of this doctoral thesis was to study the role of MRPS18-2 in cancerogenesis. Project specific aims are as follows:

1. To trace the evolutionary history of the S18 family of mitoribosomal proteins.
2. To characterize the terminally differentiated rat skin fibroblast transformed by S18-2.
3. To identify the cumulative role of RB and S18-2 in control of cell stemness and differentiation.
4. To demonstrate the status of S18-2 in human cancers, particularly colon, endometrial and prostate adenocarcinoma.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Primary cultures of REFs have been used in many studies to demonstrate the effect of different oncogenes like *Myc* and *Ras* on cell transformation. A few years ago another exciting field drew the attention of scientific community when it was reported that MEFs could be transformed into iPSCs. The set of four genes that were initially used in MEF to establish iPSC from MEF, those genes were; *SOX2*, *OCT4*, *KLF4*, and *c-Myc* [197]. Later on, iPSCs were produced from rat and human cells by expressing different combinations of genes. Since then several genes have been identified that induced the SC phenotype in primary cells.

It was shown previously in our laboratory that overexpression of S18-2 immortalized REFs [195]. The resulting cell line was termed 18IM. Expression of SC markers was observed in 18IM cells while they lost mesodermal markers. Noteworthy, 18IM cells were originally established from fibroblasts isolated from rat embryos. Thus, a major critique about this work was that primary culture probably contained some SCs of embryos, exhibiting SCs like phenotype, even though all the experiments were performed in parallel with control experiments, namely transfections with the plasmids, encoding a mutated start codons of S18-2. To clarify, S18-2 was overexpressed in terminally differentiated rat skin fibroblasts which were isolated from the skin of a mature rat. After selection with antibiotics different clones were isolated for further experiments.

3.1.1 S18-2 transformed RSFs exhibit de-differentiated phenotype

The iPSCs can develop teratomas and other kind of tumors in experimental animals [198]. Several genes and their products that induce pluripotency play pivotal roles in cell transformation and tumorigenesis. For instance, a fusion gene comprising between a portion of *OCT4* and the gene encoding N-terminus of Ewing sarcoma (EWS), a product of the chromosomal translocation t(6;22)(p21;q12) is believed to be a putative oncogene in sarcomas [199]. The 3q26–27 locus that carries the *SOX2* gene is usually amplified in solid tumors [200]. A high expression level of LIN28 is associated with epithelial ovarian cancers with poor prognosis [201] and embryonic carcinomas [202]. The LIN28 and KLF4 are also upregulated by N-Myc driven neuroblastomas [203].

Some of the clones of S18-2 immortalized RSFs also developed tumors when injected subcutaneously into SCID mice. RSFs were immortalized with GFP–S18-2 (clones 6, 13, and

17) and REFs were immortalized with pBabe–S18-2 (clones 2, 4, and 6) or GFP–S18-2 (18IM and clones 12, 10). Tumors were observed in experimental animal after injecting RSF-S18-2 clone 6 derived from RSFs. The tumors produced by REFs (clone 10) appeared after two months and with RSFs tumors were detected after three months post-injections.

3.1.2 Ectopic expression of S18-2 disrupts the normal cell cycle distribution

Stem cells have an abbreviated cell cycle with very short G_1 phase (of 2.5 to 3 hours) compared to mature cells [204]. The BrdU incorporation assay was performed for cell cycle distribution analysis. The S phase content significantly varied between immortalized cells and REFs: a higher proportion of 18IM and REF-S18-2 clone 10 cells were found in S phase compared with primary REFs. Correspondingly, the proportion of these cells in G_1 phase was reduced. In contrast, the proportions of immortalized and primary RSFs in S phase did not differ between cells. However, aneuploidy was observed in RSF-S18-2 clone 6, which was tumorigenic in SCID mice and a proportion of these cells had a DNA content of "8N". The number of cells in G_2/M was also significantly higher in RSF-S18-2 clone 6 cells than in control cells; this difference might reflect a tetraploid G_1 population.

3.1.3 S18-2 overexpression evokes chromosomal instability

Evidence of aneuploidy was demonstrated by karyotyping of RSF-S18-2 clone 6 cells and compared with REFs transformed by overexpression of *c-Myc* and mutated *Ras* (*H-Ras*) genes as well as control RSFs.

Normally, there are 20 chromosomes in rat cells and the karyotyping results indicated that control RSFs carry 20 chromosomes, c-Myc and H-Ras, transformed REF showed certain level of aberration but S18-2 overexpressed cells exhibited extremely high degree of chromosomal instability. This might explain the tumorigenic behavior of RSF-S18-2 clone 6 in experimental animals.

Previous studies reported that oncogenic transformation of REFs requires the cooperative activation of at least two oncogenes [205]. Later on it was shown that mutated Ras alone could induce the cell transformation despite its low expression in the resulting immortalized cells [206]. In same manner *c-Myc* alone could immortalize cells potently but the cells did not show characteristics of transformed cells [207]. In our study, we showed that overexpression of S18-2 alone is sufficient to transform cells, alter their cell cycle profiles, cause potential genomic instability and lead to the rise of tumors in an experimental animal model.

3.2 PAPER II

It was obvious that S18-2 play an important role in cell transformation. The next aim was to elucidate how the whole family of S18 proteins arise during the course of evolution. For this purpose, the evolutionary history of the whole S18 family was traced using bioinformatics tools and publically open databases. In total 19 bacterial and 59 eukaryotic species that represent important clades were selected for the study.

Homologs of the *Homo sapiens* S18 protein can be traced back to bacteria and they are present in all major species of cellular organisms. The consistent presence of S18 homologs in all major species of eukaryotes and bacteria indicates the functional importance and key role of S18 protein in cellular organisms.

3.2.1 Domain architecture of S18 proteins

The Ribosomal_S18 domain, a conserved sequence of approximately 152 amino acids exists in S18 proteins across all species. The domain architecture of bacterial S18 is diverse. In contrast, S18 proteins show single-domain architecture in eukaryotes. However, in S18-3 of catarrhini (old world monkeys, a group consisting of Macaca and Papio etc.) a lineage-specific protein fusion event was observed. The S18-3 protein comprised of two domains, Ribosomal_S18 and GAT_1 (Type 1 glutamine amidotransferase-like).

The conservation profile of S18 homologs suggested that despite long divergence times on the species tree, S18 orthologs are highly conserved (around 50% sequence identity on average) across bacterial species under investigation. Contrary, there are differences in the conservation profiles in metazoan S18 homologs.

3.2.2 Specific gene duplication events gave rise to three homologs of S18 in metazoans

Three rounds of duplication events have occurred in metazoan lineage; but one duplication disappeared in the branch leading to a parent of S18 of placozoa and eumetazoa. The first round of duplication resulted into S18-3 and the parent of S18-1 and S18-2. Another round of duplication took place to give rise to S18-1 and S18-2. In a recently analyzed structure of mammalian mitoribosome by Greber and co-workers, it was shown that S18-1 and S18-2 are present on small subunit while S18-3 binds to large subunit of mitoribosome [41]. The localization of S18 homologs also indicated that S18-3 was the product of an earlier gene duplication and became a part of LSU. Its sibling remained a part of SSU, where it underwent another gene duplication event to divide into S18-1 and S18-2. Furthermore, bacterial S18

shares the most sequence similarity with metazoan S18-1; this is in agreement to structural analysis which states that S18-1 occupies the position of its bacterial S18 homologs in the SSU [41].

3.2.3 The Gly132 polymorphism in S18-2

Due to the crucial role played by S18-2 in cell immortalization it was important to analyze the mutational status of S18 family proteins with the focus on evolutionarily conserved amino acids. In addition, we compared these data with the mutations of other mitoribosomal proteins in cancer. For mutational analysis of whole 55S mitoribosomal proteins in biospecimens and different cancer cell lines, the data were extracted from COSMIC database. In the S18 family, S18-1 and S18-2 were found highly mutated proteins along with other MRPs. Other highly mutated proteins of mitoribosome were MRPL32, MRPL51, and MRPL55 from LSU, and MRPS14 and MRPS25 of SSU. Interestingly, the mutation of S18-2 at Gly132 was widely observed in colon carcinoma samples. Five out of six colon adenocarcinoma (CRC) biopsies and one of ovarian carcinoma biopsies mentioned in COSMIC carried Gly132 mutation in S18-2.

To verify Gly132 mutation in *S18-2* gene the DNA was amplified from both, normal and cancer tissues of CRC patients, using primers for wild-type and mutated DNA. Amplification was detected in DNA of both normal and tumor tissue with both mutated and wild type primer. For confirmation, the PCR products were sequenced that also supported the PCR analysis. The mutation resulted in substitution of Glycine to Cysteine at position 132. This suggests the presence of a polymorphism of *S18-2* gene in CRC patients where one allele codes the Glycine and other allele codes for Cysteine. Detection by currently available technologies, 20% to 50% of CRC cases failed to show any mutations, although major genes for polyposis and nonpolyposis familial CRC have been identified. It is estimated that heredity is responsible for approximately one-third of the susceptibility to CRC [208], and that causative germ-line mutations account for less than 6% of all CRC cases [209].

Recent approaches used for the discovery of new cancer-associated genes are based on genome-wide technologies. Based on the International HapMap Project data, patterns of polymorphisms in candidate and other anonymous genes were identified that were spread throughout the genome [210].

3.3 PAPER III

For the first time the expression pattern of S18-2 was analyzed in cancer clinical specimen using endometrial cancer (EC) tissues from patients. The expression of S18-2 was analyzed in different grades of EC to evaluate the possible role of S18-2 in cancer. Samples were collected from women who underwent surgery at Karolinska University hospital. Based on the morphological features, ECs were graded according to the criteria of the 2009 International Federation of Gynecology and Obstetrics (FIGO) [11]. Samples were divided into six groups; 1. highly differentiated adenocarcinoma (HDA), 2. moderately differentiated adenocarcinoma (MDA), 3. low differentiated adenocarcinoma (LDA), 4. serous cancers (SC); 5. hyperplasia (HP) and 6. normal epithelia (NE). In total 49 samples were analyzed.

3.3.1 Expression of S18-2 protein was elevated in tumors

The S18-2 signal was not detected in normal tissues and very weak signals were observed in hyperplasia. In comparison, tumor cells demonstrated strong cytoplasmic S18-2 signal. In LDA samples a proportion of cancerous cells showed nuclear signal of S18-2. However, the proportion of such expression was less than 3-5% of all tumor cells. The statistical analysis of the staining showed a significant difference in expression levels of S18-2 protein among the group of tumor samples and normal tissue, together with hyperplasia. Surprisingly more advanced endometrioid adenocarcinomas showed a tendency of decreased S18-2 signal. The C-terminus of E2F1 (residues 409-426) is involved in binding to the RB protein, Therefore, we employed a mouse monoclonal antibody directed against the C-terminus of E2F1 protein with hope that it could recognize only the E2F1 free from RB. The nuclear signal of free E2F1 was elevated in less differentiated tumors and it was significantly higher than in normal tissue and in hyperplasia. Noteworthy, the high E2F1 expression significantly correlated with highly expressed S18-2 in analyzed tumors.

3.3.2 Ectopic expression of S18-2 is associated with EMT in EC cells

Multinucleated cells were observed when S18-2 was overexpressed in different cancer cell lines such as breast cancer cell line MCF7 and kidney tumor cells KRC/Y [211]. To monitor the molecular effect of high expression of S18-2 on EC, a cell line of EC, HEC1-A was by transfecting a GFP vector expressing full length of *S18-2* cDNA. With overexpression of S18-2 the expression of pan-keratin, cytokeratin 18, beta-catenin, and E-cadherin was decreased while vimentin was expressed more compared to parental cells. Such expression pattern of proteins indicates that cells underwent EMT upon overexpression of S18-2 that is associated with invasive ability of tumor cells.

3.3.3 Expression of S18-2 EC cells increased *in vivo* proliferation in experimental model

Both parental and S18-2 overexpressing cells were injected subcutaneously into SCID mice, in order to characterize the tumorigenicity of these cells. Tumors were detected in all injection sites (4 for each cell line) but with different proliferation rates. All experimental animals were sacrificed at the same time, day 23, when the largest tumor was observed. Growth curves showed that compared to the control HEC-1-A cells, the sub-line that expressed S18-2 constitutively at the high levels proliferated faster.

The expression pattern of S18-2 in EC clinical biospecimen, induction of EMT in EC cell and increased *in vivo* proliferation indicates the putative role of S18-2 in development of cancer.

3.4 PAPER IV

Prostate cancer was used as another model to study the role of S18-2 in tumorigenesis. As described several times, overexpression of S18-2 induced expression of SC markers. In this study the correlation between S18-2 and the SC marker CXCR4 was studied in PCa. The S18-2 expression was analyzed in 12 prostate clinical specimens with Gleason score more than 6 and compared with 11 control specimens. The neoplastic lesions in the PCa samples were highly positive for S18-2 while benign gland and hyperplastic sections were negative for S18-2 signals. CXCR4 expression was also analyzed in some of the PCa serial sections and it was found that S18-2 and CXCR4 were expressed in the same lesions.

To study the molecular effect of S18-2, sublines of PCa cell line PC3 were generated with transfection of a pBabe vector carrying full length of *S18-2* cDNA. After selection two clones were generated PC3-S18-2-CL03 and PC3-S18-2-CL04 where PC3-S18-2-CL04 cells expressed higher levels of S18-2 than PC3-S18-2-CL03.

3.4.1 S18-2 overexpression induced EMT and motility in PCa cells

The levels of EMT markers were correlated with the expression level of S18-2. E-cadherin was reduced significantly in PC3-S18-2-CL04, compared to PC3-S18-2-CL03 and PC3. The expression of cytokeratin 8 was also decreased in PC3-S18-2-CL04, in comparison to PC3-S18-2-CL03 and PC3. Though beta catenin remained unchanged but expression of Pan-keratin was lowered both in PC3-S18-2-CL03 and PC3-S18-2-CL04. Such expression pattern indicates that EMT was induced in PC3-S18-2-CL04 at a higher degree compared to PC3-S18-2-CL03 and PC3.

The mRNA expression level of EMT related transcription factors was assessed, both with overexpression of S18-2 in PC3-S18-2-CL03 and in PC3-S18-2-CL04 as well as after downregulation of S18-2 in PC3. The expression levels were compared to those in the PC3

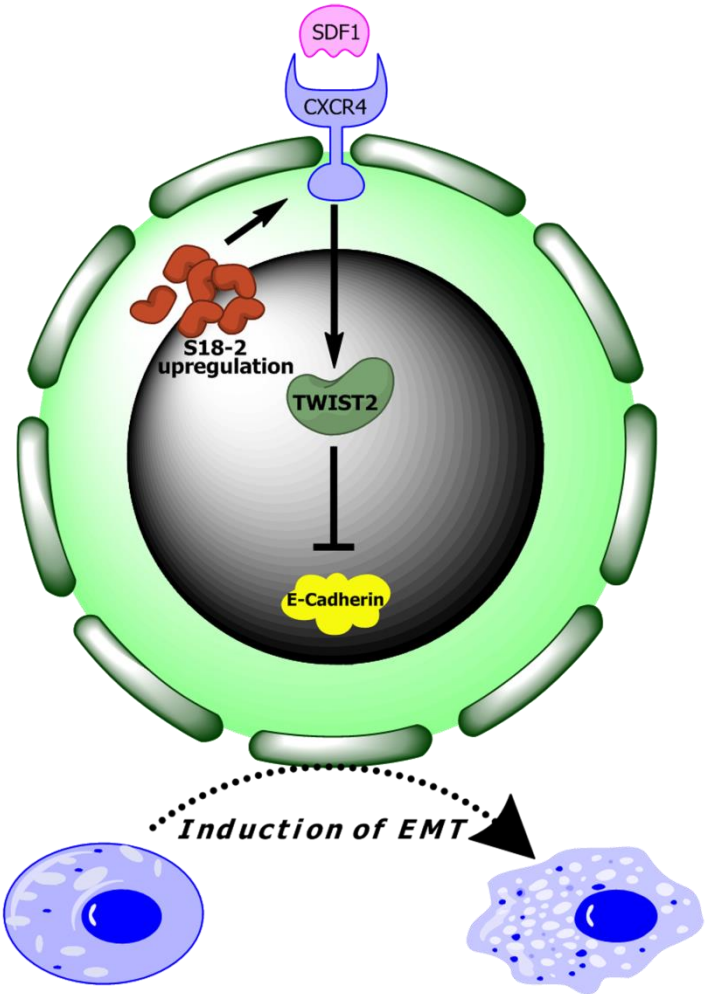

Figure 7: The Figure depicts that S18-2 can enrich the CXCR4-CXCL12 axis that upregulates the TWIST2 repressing the E-cadherin expression. Consequently, the cells undergo EMT

cells. There was no significant difference observed in the expression level of majority of thestudied genes, except for the *TWIST2* gene. The relative expression level of *TWIST2* mRNA was tightly correlated with the expression level of S18-2 protein.

To test the effect of S18-2 on the induction of EMT and migration of PCa cells, zebrafish embryos were used. The DII labeled PC3, PC3-S18-2-CL03 and PC3-S18-2-CL04 cells were injected in perivitelline space of 48 hours post fertilization (hpf). Interestingly, the number of PC3-S18-2-CL04 migrated cells was significantly higher compared with PC3-S18-2-CL03 and original PC3 cells. The number of PC3-S18-2-CL03 migrated cells lie between the values

for PC3-S18-2-CL04 and original PC3 cells. To confirm the effect of S18-2 on the CXCR4-driven migration of cells, S18-2 expression was decreased in the aggressive clone PC3-S18-2-CL04 by transfection of S18-2 specific siRNA. This in turn dramatically decreased the CXCR4 dependent migration of resulting cells when compared to same cells transfected with control siRNA.

3.4.2 CXCR4-CXCL12 axis

Prostate cancer is the neoplasm of epithelial cells but the stroma cells of prostate are also important for the progression of cancer. Generally, the communication between stroma and tumor cells is mediated through cytokines and their receptors. The CXCL12-CXCR4 axis has been a subject of many studies devoted to characterization of different tumors. The *TWIST* genes are described as master genes of EMT and that TWIST2 expression is under control of the CXCR4-CXCL12 axis [212].

The PC3 cells already expresses CXCR4 at certain level. However, with the overexpression of S18-2, a slight but statistically significant increase in the CXCR4 expression was observed in PC3-S18-2-CL04 cells. While the signal of CXCR4 expression in the PC3-S18-2-CL03 and in PC3 cells was almost similar. Following overnight starvation of the cells, the next day cells were treated with CXCL12. Significantly higher signal of migration was detected in PC3-S18-2-CL04 group, compared to PC3-S18-2-CL03 ($p=0.093$) and PC3 ($p= 0.007$). The number of cells that migrated in response to CXCL12 was in the same pattern as; the level of EMT makers, migration in zebrafish, mRNA level of EMT related TF, and the expression level of CXCR4. One of the effects of the CXCL12-CXCR4 axis in the migration of cancer cell is through increase of TWIST expression that is a repressor of E-cadherin. It was also observed in this study that S18-2 increased the CXCR4 expression and the cells underwent EMT.

To further explore the CXCR4 mediated migration upon S18-2 overexpression, CXCR4 was masked in PC3-S18-2-CL04 by anti-CXCR4 antibody which also caused significant reduction in the CXCL12 directed migration of cells compared to same cells cultured with isotype control antibody. The migration was observed in zebrafish embryo model.

3.4.3 Tumorigenesis of cells in experimental animals, SCID mice

PC3 cells proliferate faster than PC3-S18-2-CL03 and PC3-S18-2-CL04 cells. The tumor initiation was similar for all the cells. However, after 6 weeks the PC3 cells develop larger tumors compared to tumors produced by PC3-S18-2-CL03 and PC3-S18-2-CL04 cells. This

indicates that all the three-cell type exhibit the same potential of giving rise to tumors but later on the proliferation of cells with overexpression of S18-2 was reduced. The tumors produced by PC3-S18-2-CL04 contained many foci and buds which is a strong indication of metastatic tumors with more migratory ability.

These data suggest that S18-2 enriched the CXCL12-CXCR4 axis that upregulated the expression of TWIST2 which is a repressor of E-Cadherin. Consequently, due to the loss of E-Cadherin, cells underwent EMT and gained the ability to migrate more with overexpression of S18-2, as presented in Figure 7.

3.5 PAPER V

The Retinoblastoma protein controls cellular fate through governing physiological processes like cell proliferation and differentiation. Previous studies showed that RB exists mainly in the inactive phosphorylated form while only small traces of active RB are present in the nucleus of ESCs [213]. Beyond the direct control of RB in the transcription of genes through E2F1, it can also interact with chromatin remodeling enzymes. Such interactions of RB may be important for its ability to regulate the global genes expression, including the genes involved in cell stemness and differentiation [190, 214-218]. Based on the fact that there is a strong correlation between cancer and the differentiation state of cells and that S18-2 is a RB interacting protein that induces the expression of SC marker, we studied the cumulative effect of overexpression of S18-2 in the presence and absence of RB with regards to maintenance of cell stemness and differentiation. For this purpose, fibroblasts isolated from mouse embryos that were homozygously knockout for *RB1* gene were used. Three more sub lines were generated by transfection with different combinations of *S18-2* and *RB1*. Firstly, the *RB1* knockout fibroblast (RH) were transfected with plasmid expressing the GFP-S18-2 fusion protein. The resulting immortalized cell line was named as RH18. Then, the RH and RH18 cells were further transfected by a plasmid encoding the full-length RB. The individual clones produced by the transfected cells were analyzed for RB expression. Further work was performed on RH cells and 3 sub-lines: RH18 that expressed S18-2 at higher levels, RH18RB that expressed S18-2 in the presence of RB and RHRB where RB was reconstituted but with endogenous level of S18-2.

The cells ectopically expressing S18-2 in the presence of RB attained a morphology resembling mouse ESCs in culture. Several colonies were observed in RH18RB cultures, some of the colonies were round but most of them were dome-shaped of rapidly growing cells with tight borders and close packing.

The S18-2 overexpressing cells lost smooth muscle actin (SMA) both in the presence and absence of RB. Most interestingly, with the overexpression of S18-2 the RB was localized both in the cytoplasm and nucleus of RH18RB cells as shown by immunofluorescence analysis. Cell fractionation following western blot analysis confirmed the presence of RB both in the cytoplasm and nucleus.

3.5.1 Highly expressed S18-2 is sufficient to induce the telomerase activity

Stem cells have prolonged proliferative ability; they should have a mechanism to stabilize the length of telomere through several cell divisions. The ESCs and undifferentiated embryonal carcinoma cells display high levels of hTERT expression and telomerase activity. Increased telomerase activity by overexpression of TERT improved self-renewal ability, proliferation and differentiation efficiency in ESCs [219]. The deficiency of telomerase activity was observed to be related to DNA methylation and histone deacetylation of *TERT* gene in differentiating ESCs [220]. Therefore; high levels of telomerase activity or the expression of TERT are believed to be a marker of pluripotent ES cells [221].

High telomerase activity was associated with overexpression of S18-2 in RH18 and RH18RB cells. The expression of RB with endogenous levels of S18-2 in RHRB cells resulted in marked decrease of telomerase activity. It has been shown that replicative senescence is linked to the RB and its interacting partners. Indeed, activation of both the p53 and p16INK4A–RB pathways are important for the induction of senescence in a variety of human cancers [222]. Regardless of RB expression the overexpression of S18-2 enhanced the telomerase activity that is an important prerequisite for the proliferation of SCs.

3.5.2 S18-2 overexpressed cells with reconstituted RB behave like SCs

The RT^2 profiler array is a qPCR based efficient approach to study the expression pattern of genes involved in different cellular processes. The mRNA expression level of 84 genes, specific for mouse ESCs in the four cell types were assessed. The genes represented: ESC markers, chromatin modification and remodeling factors, transcriptional regulators, transcription factors, markers of pluripotency, iPSC enhancers, markers of ectoderm, mesoderm and endoderm lineage, pre-adipocytes progenitor cell markers and specific markers of neural, cardiac, early smooth muscle, early endothelial, hematopoietic and mesenchymal SCs.

Most of the mouse ESC related genes were up regulated in RH18RB cells compared to the other three cell types. Noteworthy, the *Gata-1* gene, was highly expressed in RH18RB cells compared to RH cells. *Gata-1* is the marker specific for endothelial cells.

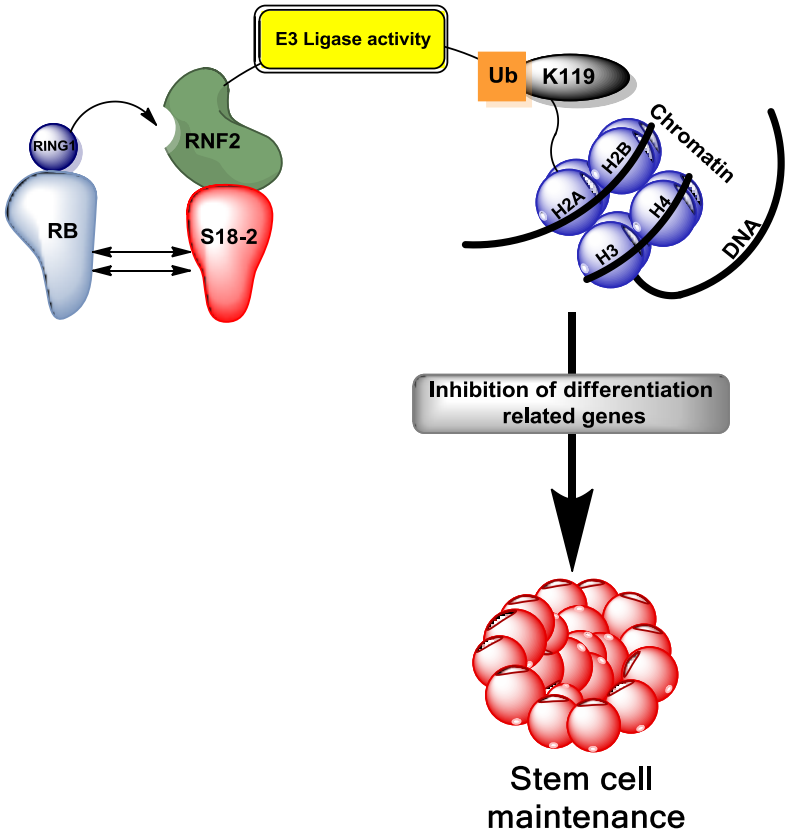

Figure 8: S18-2 binds RNF2 while RB interacts with RING1, the interaction between S18-2and RB bring RNF2 and its adopter RING1 in close proximity, leading to increased activity of RNF2. Consequently, histone H2A is highly monoubiquitinated at lysine 119 that maintains the SCs phenotype**.**

The GATA-1 protein is essential for the development of megakaryocytic (platelet producing cell) and erythroid cells (red blood cell) [223]. It helps in transcription of the α-spectrin [224], an important structural protein of red blood cells. Interestingly, the GATA1 is an endothelial cell marker but the origin of RH18RB cells (which was originally MEF) is mesenchymal. It indicates that RH18RB cells demonstrate mesenchymal cells characteristics but might also show traits of the endodermal and ectodermal cell lineages. Another gene that was highly expressed in RH18RB cells was the *Thy* compared to RH cells. *Thy1 or CD90* is a marker of HSC, Thy-1 has been postulated to be involved in cell adhesion, cellular recognition and T-cell activation [225].

The expression of SOX2 and OCT4 was observed at protein level in S18-2 overexpressed cells. The expression levels of these markers were higher in RH18 cells than RH18RB. Presumably, in RH18RB culture due to presence of RB, a proportion of cells spontaneously

differentiated, while RH18 cells might lack the differentiation ability. The directed differentiation method is widely used to test the pluripotency of SCs *in vitro*. To study the role of highly expressed S18-2 and RB in the pluripotency of cells, the four cell lines were subjected to directed differentiation using cocktails of chemicals.

Dexamethasone, ascorbic acid 2-phosphate and glycerol 2-phosphate were used to induce osteogenesis. It has been reported that dexamethasone induced *Runx2* expression through FHL2/β-catenin-mediated transcriptional activation. Moreover; dexamethasone increased Runx2 activity by upregulation of tafazzin and MAP kinase phosphatase 1. Ascorbic acid increases the secretion of collagen type I (Col1), which in turn leads to increased Col1/α2β1 integrin-mediated intracellular signaling. The glycerol 2 phosphate provides a source of phosphate in hydroxylapatite and in addition influences intracellular signaling molecules [226]. The robust effect of this cocktail of chemicals leads to differentiation of pluripotent SCs to bone forming progenitor cell, the osteoblasts. To stain osteoblast in cell cultures Alizarin red S solution was used. The Runx2 is the main transcription factor regulating the osteogenic differentiation that was upregulated in RH18RB cells after differentiation induction. Furthermore, *Osp* was also highly expressed in RH18RB cells that is one of the Runx2 responsive genes and is also believed as marker of osteogenesis. The Osp helps in bone remodeling via integrin mediated pathway in osteoblasts and osteoclasts by directly binding to integrin αVβ3 [227].

After treatment for the induction of chondrogenic differentiation, cells were treated with acidic solution of Alcian blue that stained the proteoglycans of chondrocytes. Sox9 is the transcription factor that regulates the chondrogenic differentiation. Sox9 controls the expression of aggrecan that is a critical component for cartilage structure and carries the binding domain for hyaluronic acid (HA). Aggrecan plays an important role in mediating chondrocyte-matrix and chondrocyte-chondrocyte interactions through its ability to bind HA and linker proteins. We showed that in the presence of RB and S18-2 overexpression (RH18RB cells) Sox9 expression was elevated both at mRNA and protein level.

Previously it has been reported that RB promotes the differentiation of multiple lineages by monitoring the activity of master transcription factors such as MyoD in muscles [207], Runx2 in bones [228], and PGC-1 in adipocytes [229]. However, RB individually as well as highly expressed S18-2 alone was not sufficient to drive the differentiation of SCs. But the presence of RB simultaneously with highly expressed S18-2 could drive the differentiation of SCs to different cell lineage.

3.5.3 Tumorigencity of cells

The RH and RHRB cells failed to grow even after 20 weeks of injection. Both RH18 and RH18RB cells produced tumors in mice, RH18RB cells were highly tumorigenic as tumor appeared with in three weeks in RH18RB injected mice. The tumor initiated very quickly and they were also larger in size (14 mm^3) compared to RH18 tumors.

A portion of RH18 tumors appeared like epithelial cells and few necrotic cells were also observed in those tumors. RH18RB tumors were more aggressive and without necrotic cells in tumors. The RH18RB tumor cells expressed Pan-keratin and very few cells showed strong expression of cytokeratin 18. They lost the expression of vimentin.

3.5.4 S18-2 and RB maintain cell stemness and differentiation by enhancing the RNF2 activity

Using the GST pulldown assay it was demonstrated that S18-2 can bind to Rnf2. The effect of this association was analyzed with high expression and endogenous expression of S18-2 both in the presence and absence of RB. The Rnf2 was expressed at the same level in all four cell types. The activity of Rnf2 was assessed by analyzing the levels of Ub-K119 H2A. The highest activity of Rnf2 was detected in RH18RB cells, moderate levels in RHRB cells but relatively lower in RH18 cells while Rnf2 was inactive in RH cells. It indicates that Rnf2 needed RB for its function but the highest activity was observed with the overexpression of S18-2 and RB together.

Polycomb group proteins (PcG) are well known for their control of gene expression via chromatin modification during development [230]. The PcG is an assembly of several proteins, in the form of two complexes: polycomb repressor complex (PRC) I and II. The PcG proteins regulate gene expression with a repressive function that is stable over many cell generations. The Ring1B or Rnf2 is one of the members of PRC-I, it has been shown to interact with and silence the activity of CP2 (TFCP2/CP2) transcription factors [231]. The role of Rnf2 has been studied in different cellular processes like embryogenesis, development, self-renewal, differentiation and cancer. Rnf2 is an ubiquitin E3 ligase enzyme; it suppresses the expression of target genes through mono-ubiquitination of lysine 119 of histone H2A (Ub-K119-H2A). RING1, another protein of PRC1 act as modulator of RNF2 and it is required for RNF2 activity.

It has been shown that Rnf2 contributes to stable maintenance of mouse ESCs. Rnf2/Oct4/Nanog could bind to 212 common genes, with the deficiency of RinG1B the

expression of 25 genes was found altered [116, 232, 233]. Out of the 25 genes 18 were de-repressed following the knockout of Rnf2 in mouse ESCs. This suggests that Rnf2 is required for the maintenance of undifferentiated and pluripotent SCs by repression of specific subsets of Oct4 and Nanog associated genes. Some of the Nanog and Oct4 co-occupied 18 genes were *Gadd45g, Fgf15, Bmp7, Col4a2, Podxl, Gata3, Bmi1, Msx2, Gja1* and *Eif4g3* [233].

The Rnf2 was found completely inert in the absence of RB with endogenous expression of S18-2 (RH cells). Lower activity of Rnf2 was associated with the expression of RB at physiological level of S18-2. Even some activity of Rnf2 was observed with S18-2 overexpression alone in the absence of RB, but Rnf2 was highly active with the overexpression of S18-2 and RB (RH18RB cells), together.

These data suggest that RB binds to RING1 and S18-2 interact with Rnf2, but RB and S18-2 also interact with each other which might bring RING1 and Rnf2 in close proximity. Such interactions result in highly ubiquitination of H2A which is important for stem cell identity as shown in Figure 8. The deficiency of Rnf2 resulted in the embryonic lethality and caused the gastrulation arrest as well as cell cycle inhibition [234]. Furthermore, Rnf2 is required for chondrocyte and osteocyte differentiation in zebrafish by stabilizing the expression of transcription factors *RUNX2* and *SOX9* that are needed for the differentiation of these lineages [235]. It has been found that Rnf2 raised the expression of *Sox9* by inhibiting the expression of its repressor, the *Msx2* [233, 236]. We also reported in the current study that overexpression of S18-2 and RB; increased the Rnf2 activity, induced the differentiation and the expression of both *SOX9* and *RUNX2*. And knockdown of S18-2 in zebrafish embryos induces embryonic lethality.

3.5.5 Role of S18-2 in development of zebrafish embryos

The S18-2 expression in zebrafish embryos reached a maximum level in the period of 4–6 hpf. Knockdown of the S18-2 protein in zebrafish embryos induced embryonic lethality. Moreover, the embryos exhibited a severely abnormal phenotype: they were significantly smaller in size, their endoderm was underdeveloped, and segmentation did not occur. In zebrafish embryos, the *S18-2* gene was disrupted via transposon mutagenesis that resulted in truncation of first 75 residues of corresponding protein. The fish died at post-fertilization day 10 because of impaired cardiac contractility [237]. The function of S18-2 in embryogenesis should be further elucidated.

3.6 PAPER VI

The S18-2 immortalized REFs (18IM) which are described above were used in this study. 18IM cells showed all the characteristics of SCs but unlike SCs they failed to give rise to tumor in experimental animals. To study such asymmetrical behavior of these cells they were challenged to NK cell mediated cytotoxicity and directed differentiation in vitro.

It has been shown that NK cells target the embryonic SCs or cancer initiating cells compared to differentiated cells. One of the reason is their reduced expression of MHC I proteins. It is reported that ESCs and cancer initiating cells expressed MHC I at very low levels [238]. 18IM cells also showed very low expression of MHC I molecule compared to control REF. *In vitro*, 18IM cells were presented to IL-2 activated rat splenocytes of same species from which the parental REFs were previously isolated to generate the 18IM cells. They were better recognized and killed compared to control REF as measured by chromium release assay. When the NK activating receptor of these splenocytes was blocked by antibody, 18IM killing was reduced. The similar behavior was observed when NK cells were used from SCID mice in which 18IM cells failed to produce tumors. The chemokine, cytokine and their receptor was measured most of those molecules that raised the cytotoxic activity of NK cells were upregulated at mRNA level in 18IM cells compared to REFs. Finally, the NK cells were shown by to be recruited *in vivo* to the site of 18IM injection.

Another possibility of asymmetric behavior might be the trans-differentiation of SCs like 18IM cells. *In vitro* 18IM cells challenged for directed differentiation to different cell lineages like osteogenesis, chondrogenesis. Probably the cells acquired all the factors required for differentiation and trans-differentiated instead of proliferation.

Concluding, the overexpression of S18-2 enhance the tumorigenesis and evoke NK cell cytotoxicity due to decreased MHC I expression. S18-2 might be selected for targeted therapy against cancer.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

In the light of results described in the current doctoral thesis, we characterized the role of MRPS18-2, a RB interacting protein, in the control of several cellular processes, including proliferation, stemness and differentiation.

The S18-2 protein belongs to the S18 family, which includes a group of proteins, conserved through evolution. We described the "rise" of S18 proteins from bacteria to metazoans, identifying conserved residues in all the three S18 proteins. These finding might be helpful to find the normal physiological functions of S18 proteins which are still unknown. Phylogenetic analysis of S18 proteins revealed specific gene duplication events that resulted in three S18 homologs in metazoan.

We found that overexpressed S18-2 can immortalize the terminally differentiated primary rodent cells. These cells show chromosomal instability, enhanced telomerase activity and tumorigenecity in SCID mice. The rodent cells immortalized by overexpression of S18-2 were targeted by NK cell mediated cytotoxicity. The NK cell immunogenicity make S18-2 a putative target for future therapies against cancer.

The differential expression of S18-2 in endometrial cancer, prostate cancer and a consensus Gly132 polymorphism of S18-2 in colon cancer enlighten the role of S18-2 in tumorigenesis. We demonstrated that S18-2 can induce EMT in EC and PCa. Moreover, overexpressed S18-2 induced the CXCR4 mediated migration of PCa cells in *vitro* and in a zebrafish model. Our findings describe the new mechanism of cell migration induced by S18-2 overexpression as a putative molecular mechanism underlying metastasis.

We revealed the new functional consequences of RB-S18-2 interaction in the control of cell stemness and differentiation. A cytoplasmic protein complex between S18-2, RB, and the RNF2 was detected. Such interactions enhanced the E3 ligase activity of RNF2, thus, maintaining cell stemness. This finding may open a new insight in the regulation of differentiation which is a major challenge in the application of stem cell biology.

The data described in this doctoral thesis strongly suggest that S18-2 plays an important role in the development of cancer as a potent oncoprotein. Important questions for the future include: 1) Elucidation of the physiological function of S18-2; 2) Understanding the role its CXXC zinc finger DNA binding motif; 3) Analyzing the status of S18-2 in other human tumors, particularly in tumors where RB is deleted or mutated; 4) The binding between S18-2 and RB at different stages of cell cycle should be analyzed as it may reveal a novel

mechanism of cell cycle regulation; 5) The role of S18-2 in embryogenesis must be further studied.

The justification of these studies from a cancer therapy perspective is that S18-2 could be an attractive target for development of future cancer therapies.

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